Charles University in Prague
Faculty of Medicine in Hradec Králové

Medical Chemistry and Biochemistry

Molekulární detekce invazivních mykotických onemocnění u imunokompromitovaných pacientů

Molecular detection of invasive fungal disease in immunocompromised patients

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Hradec Králové, 2013 Defence on: .....

DECLARATION

I declare hereby that this dissertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University in Prague, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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The work presented in this thesis is a summary of my five years fellowship from February 2001 to February 2006 at the Division of Molecular Microbiology and Development of Genetic Diagnostics at the Children's Cancer Research Institute in Vienna, Austria under the supervision of Prof. Thomas Lion.

The focus of my work is on the development of molecular diagnosis of invasive fungal disease in immunocompromised patients. Together with Mag. Sandra Preuner and Mag. Christine Landlinger I have established three DNA-based detection assays for the detection and identification of a large number of the clinically relevant fungal species.

The development of the methods as outlined would not been possible without the contribution of Prof. Birgit Willinger and Assoc. Prof. Dr. Vladimír Buchta, who cultivated and donated a number of various common and rare fungal cultures and clinical probes for the laboratory testing.

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V dizertační práci se mi podařilo vyvinout tři PCR metody pro kvantitativní detekci a identifikaci kvasinkové a plísňové DNA. Dvě metody založené na kvantifikaci v reálném čase s názvem PanAC PCR a “panfungal” PCR byly navrhnuty tak, aby detekovaly a kvantifikovaly široké spektrum plísní a kvasinek způsobujících invazivní mykotická onemocnění. Význam metod pro klinické využití byl v rámci standardizace testován retrospektivně na souborech pacientů s již dokumentovanými invazivními mykózami a dále pak prospektivně na souborech pacientů s vysokým rizikem invazivní mykózy.

Vzhledem k významu přesné identifikace původce onemocnění byla vyvinuta “semi-nested” PCR s fluorescenční detekcí pomocí kapilární elektroforézy umožňující rychlou identifikaci plísně či kvasinky v klinickém materiálu, který byl pozitivní v jedné ze širokospektrých screeningových PCR. Možnost klinického využití této metody byla taktéž testována na populaci pacientů s dokumentovanou invazivní mykózou.

SUMMARY

Molecular detection of invasive fungal disease in immunocompromised patients

In my work I have been able to establish three different PCR-based assays for the quantitative detection and identification of fungal DNA. Two DNA-based detection assays termed PanAC PCR and panfungal PCR based on the real-time quantitative (RQ-PCR) technology were designed to detect and quantify the most important fungal genera currently associated with IFD including a large number of pathogenic moulds and yeasts. Upon standardization of both RQ-PCR techniques, the applicability in the clinical setting was assessed by investigating a series of clinical specimens from patients with documented fungal infection, and by prospectively studying patient cohorts at high risk of IFD.

In view of the importance of precise identification of the causative fungal pathogen, a semi-nested PCR method coupled with fluorescent capillary electrophoresis detection was established. It facilitates rapid identification of fungal species in clinical materials that test positive for IFD using one of the broad-range screening assays. This method was also tested in a population of patients with documented fungal infections to assess its clinical potential.

ABBREVIATIONS

A	Adenine
AB	Applied Biosystems
Ab	Antibody
Ag	Antigene
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline proteinase gene
Asp	*Aspergillus*
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BDG	1,3-β-D-glucan
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
C	Cytosine
Can	*Candida*
Cdc2	Cell-division-cycle molecule 2
cDNA	Copy deoxyribonucleic acid
CFU	Colony forming unit
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
C_T	Threshold cycle
Cu-ZnSOD	Copper-zinc superoxide dismutase
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded deoxyribonucleic acid

DSM	German Collection of Microorganisms
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EORTC	The European Organization for Research and Treatment of Cancer
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FAM	Fluorescein amidite (6-carboxyfluorescein)
FDA	Food and Drug Administration
FMCU	Faculty of Medicine, Charles University
Fw	Forward
G	Guanine
gDNA	Genomic DNA
GM	Galactomannan
gp43	43 kDa glycoprotein
HR-CT	High resolution computed tomography
IA	Invasive aspergillosis
IC	Invasive candidiasis
IFD	Invasive fungal disease
IFI	Invasive fungal infection
IFICG	Invasive Fungal Infections Cooperative Group
IGS	Intergenic spacer
IHMM	Institute of Hygiene and Medical Microbiology
ITS1	Internal transcribed spacers 1
ITS2	Internal transcribed spacers 2
KOH	Potassium hydroxide
LLB	Lyticase lysis buffer
LNA	Locked nucleic acid
LSU	Large subunit

MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
$MgCl_2$	Magnesium chloride
MSG	Mycoses Study Group
NaCl	Sodium chloride
NASBA	Nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
Pan-AC	Pan-*Aspergillus* -*Candida* assay
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-ELISA	Polymerase chain reaction with enzyme-linked immunosorbent assay detection
pH	Measure of the activity of the (solvated) hydrogen ion
R	Guanine or adenine
rDNA	Ribosomal deoxyribonucleic acid gene
Rev	Reverse
RFLP	Restriction fragment length polymorphism
RFUs	Reference fluorescence units
ROX	6-carboxyl-X-rhodamine
RPMI	Roswell Park Memorial Institute medium
RQ-PCR	Real-time quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid gene part
SAP(s)	Secreted aspartic proteinase gene(s)
SSU	Small subunit
T	Thymine
TAMRA	6-carboxytetramethylrhodamine
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
UNG	Uracil N´-glycosylase
ΔRn	Fluorescence intensity of the reporter
Y	Cytosine or thymine

PREFACE

Invasive fungal diseases (IFDs) have become an increasingly common life-threatening complication in a variety of critically ill patients resulting in high mortality rates. The changes in treatment strategies, particularly the implementation of fungal prophylaxis in high-risk patients, have resulted in remarkable changes in the spectrum of fungal pathogens causing IFDs. Yeast infections have shifted towards a higher proportion of non-*albicans Candida* species but their overall incidence has remained stable. In contrast, IFDs caused by moulds including particularly various species of *Aspergillus*, *Fusarium* and Mucorales have increased in number. Current diagnostics of IFD is based primarily on conventional approaches such as microscopy, cultivation, histopathological examination and imaging methods which still represent the gold standard. The diagnosis, however, remains difficult because of the limited sensitivity and specificity of these methods. The detection of surrogate serological markers such as the fungal cell wall components galactomannan and (1,3)-β-D-glucan by commercially available diagnostic kits has become increasingly important and widely used, but the clinical interpretation of the results still remains controversial.

Rational and timely initiation of adequate antifungal treatment is of paramount prognostic importance, but the lack of sufficiently rapid and reliable diagnostics is a major impediment. In the clinical setting, the onset of antimycotic treatment is therefore generally based on indirect evidence for systemic fungal infections: i.e. clinical signs of sepsis unresponsive to different antibacterial agents for greater than 72-96 hours. This strategy, however, may lead to a significant and possibly deleterious delay of antimycotic therapy in patients with IFD. On the other hand, the pronounced organ toxicity of some antimycotic drugs provides a strong argument against prophylactic application of antifungal agents. Initiation of systemic antimycotic treatment based merely on clinical suspicion results in an unnecessary increase of organ toxicity and costs in instances in which no IFD is present. It is of great interest therefore to establish

ways of managing antimycotic treatment based on firm evidence. To address this task, it is necessary to employ diagnostic approaches permitting rapid, sensitive and specific detection of IFDs. This would allow timely initiation of treatment, which is crucial for favorable outcome. Moreover, the possibility of identifying the causing fungal pathogen would greatly contribute to optimal therapeutic decisions.

Recently, a plethora of PCR-based diagnostic methods targeting different gene regions and exploiting a variety of amplicon detection tools was published and demonstrated the possibility to overcome the limitations of other diagnostic approaches. However, the current lack of methodological standardization and validation has precluded clear interpretation of the results and prevented broad application of PCR-based fungal diagnostics in the clinical setting.

The present work addresses the current dilemma in molecular diagnostics of IFD: two DNA-based detection assays termed PanAC PCR (see chapter 2.1) and panfungal PCR (see chapter 2.2) based on the real-time quantitative (RQ-PCR) technology have been established. The PanAC assay was designed to detect and quantify the most important fungal genera currently associated with IFD including a large number of pathogenic *Candida* and *Aspergillus* species in a single reaction. The panfungal assay was developed to cover essentially all clinically relevant fungal species in two different reactions, one focusing primarily on moulds and the other on yeasts and Zygomycetes. More specifically, the spectrum of human pathogenic fungi covered by these assays includes all important *Aspergillus* and *Candida* species, as well as other yeasts and moulds of potential clinical relevance, such as *Trichosporon*, *Cryptococcus, Fusarium*, *Mucor* or *Rhizopus* species, and even extremely rare pathogens such as *Saccharomyces cerevisiae* and *Malassezia furfur*. The conceptual design of the RQ-PCR assays presented in this work has significantly extended the capacity of RQ-PCR approaches existing at the time of publication by facilitating the detection of more than 16 (PanAC assay) and >80 (panfungal assay) different pathogenic fungal species, respectively. Upon standardization of both RQ-PCR techniques, the applicability in the clinical setting was assessed

by investigating a series of clinical specimens from patients with documented fungal infection, and by prospectively studying patient cohorts at high risk of IFD (see chapters 2.1 and 2.2).

Moreover, in view of the importance of precise identification of the causative fungal pathogen, a semi-nested PCR method coupled with fluorescent capillary electrophoresis detection was established (see chapter 2.3). It facilitates rapid identification of fungal species in clinical materials that test positive for IFD using one of the broad-range screening assays. This method was also tested in a population of patients with documented fungal infections to assess its clinical potential (see chapter 2.3).

The entire work outlined above was published in four scientific papers which constitute the backbone of the doctoral thesis presented. Three of the papers focus on the respective methodologies and their clinical application, and one reviews the current status of molecular diagnostics in IFD. The thesis is divided into three sections with overlapping description of methodological aspects (e.g. fungal DNA extraction procedures from various types of clinical specimens or fungal strains employed in testing), but the specific methodologies and results of clinical analysis are described and discussed in individual sections.

It should be pointed out that the novel molecular diagnostic assays including the panAC and the panfungal PCR method have been patented (No. 06817468.9– Europe) prior to publication, and negotiations with potential industrial partners for the production of commercial diagnostic kits are ongoing.

1. INTRODUCTION

Invasive fungal diseases (IFDs) continue to represent a significant problem in a large proportion of critically ill patients, particularly in individuals with impaired immunity. Prolonged episodes of profound neutropenia represent a high-risk constellation primarily in patients with hematologic malignancies (Michallet and Ito 2009), recipients of allogeneic bone marrow (Badley et al. 2001), peripheral hematopoietic stem cells (Marr 2010) or solid organ transplants (Person et al. 2010). Moreover, AIDS patients (Hage et al. 2002) or individuals with other acquired immunodeficiency conditions (Pappas 2010), intensive care patients (Smith and Kaufmann 2010), preterm neonates (Kaufman 2007) and patients undergoing long-term corticosteroid therapy (Lionakis and Kontoyiannis 2003) also carry a high risk of IFD. In addition to the state of immunosuppression, long-term exposure to broad spectrum antibiotic therapy, high-dose anticancer chemotherapy, long presence of indwelling catheters, and biological factors such as iron overload and patient age (Erjavec et al. 2009; Miceli et al. 2006) have also been shown to increase the risk of developing IFD. Furthermore, recently recognized genetic factors predisposing to IFD, such as impaired NADPH-oxidase activity (Segal and Romani 2009), disturbed production of tumor necrosis factor α or interleukin-10 (Sainz et al. 2007a,b) and genetic polymorphisms in Toll-like receptors that result in deficient production of some inflammatory cytokines (Bochud et al. 2008) represent conditions constituting a group of vulnerable patients at high risk for IFD.

The morbidity and mortality of IFD generally depend on the causative pathogen, geographical location and underlying patient condition. Since the symptoms of IFD are often non-specific, fungal pathogens are not identified as causative agents in many instances or the diagnosis is established late in the course of infection. This contributes to high overall mortality rates ranging between about 30-80% (Lass-Flörl 2009), and even exceeding 90% with central nervous system involvement and dissemination (Walsh et al. 2008). Although the vast majority of these life-threatening infections are still attributable

to *Candida* and *Aspergillus* species, shifts in epidemiology have been noted with the adoption of antifungal prophylaxis resulting in the increasing occurrence of resistant species or the emergence of previously rare fungal species displaying inherent resistance to antifungal agents used in the prophylactic setting (Preuner and Lion 2009). Recent studies indicate a slight decrease of IFDs due to *Candida albicans*, while the incidence of non-*albicans Candida* infections, including those with reduced antifungal susceptibility (e.g. azole- and/or echinocandin-resistant *C. glabrata, C. krusei, C. parapsilosis* and *C. albicans*) is rising (Michallet and Ito 2009; Lass-Flörl 2009). In addition, invasive aspergillosis (IA) has surpassed invasive candidiasis (IC) as the most common IFD encountered in the peripheral hematopoietic stem cells transplant population (Person et al. 2010). Although *A. fumigatus* continues to be the most common cause of IA, non-*fumigatus Aspergillus* species occur with increasing frequency. Of particular relevance is *A. terreus* which is associated with amphothericin B resistance and higher mortality (Lass-Flörl 2009). A similar spectrum of fungal pathogens is also observed in pediatric patients but with different frequency and mortality rates. The incidence of IC is higher in children than in adults (47 vs. 30/100,000 admissions) with a mortality rate of 10%, whereas the frequency rates of IA are similar in children and adults, with mortality rates between 30 and 50% depending on the underlying disease (Dornbusch et al. 2009). Beside IFDs caused by *Aspergillus* and *Candida* species, hitherto rarely observed fungal genera associated with very high fatality are becoming more common (Lass-Flörl 2009; Richardson and Lass-Flörl 2008). These include hyalohyphomycetes such as *Fusarium* and *Scedosporium,* phaeohyphomycetes (darkly-pigmented or dematiaceous fungi), basidiomycetous yeasts (*Cryptococcus*, *Trichosporon*, *Malassezia*), and, very importantly, different members of the Mucorales, namely *Rhizopus* and *Mucor*. IFDs are often difficult to diagnose, but early detection of the causative pathogen is an essential prerequisite for timely onset of appropriate antifungal therapy and favorable outcome. Therefore, the development of rapid and reliable methods for early diagnosis of IFDs is urgently required to improve clinical management of the disease.

1.1 Conventional diagnostic tests

Current conventional diagnostic methods for IFDs are based on microbiological examination and imaging techniques. In general, microscopy, cultivation and most serological tests show inadequate sensitivity or specificity, and are often not fast enough to permit rational and timely initiation of treatment. Combination of these methods can overcome of these shortcomings and achieve more satisfactory results.

1.1.1 Microbiological examination

Microscopy-assisted examination of microbiological samples or histological material enables the detection of fungal structures only when their abundance in the sample is high. In addition, positivity in biopsy samples is usually associated with an advanced stage of fungal infection (Cuenca-Estrella et al. 2011). Histopathological examination of targeted biopsies of suspicious lesions identified by imaging methods provides an important improvement in the diagnosis of IFD, but is frequently hampered by the risk of bleeding complications in patients with severe thrombocytopenia (Denning 1998). Another way how to increase sensitivity of microscopical examination is using fluorescent dyes (e.g., calcofluor) which are specifically binding to components of fungal cell wall, including *Pneumocystis jiroveci.* In case of histopathological sections immunohistochemical techniques can be useful in discrimination among fungal pathogens, e.g. *Aspergillus* spp. from other filamentous fungi such as *Fusarium* or *Scedosporium* (Choi et al. 2004; Kaufman et al. 1997; Verweij et al. 1996).

Mycological cultivation of various biological specimens including peripheral blood has been routinely employed for the detection of clinically relevant fungal pathogens. However, blood cultures were described to fail in approximately 50% of infections reported in patients with IC detected at autopsy (Pemán et al. 2011). In addition, aspergilli and other moulds are rarely diagnosed from blood cultures

with exceptions such as *Fusarium* species. Negative findings therefore cannot readily exclude IFD (Simoneau et al. 2005).

1.1.2 Identification

Identification of clinically relevant fungi at the species or genus levels plays an increasingly important role as the spectrum of human pathogenic fungi broadens. It can provide additional important information on the causative agent and the nature of infection especially in terms of its course, intensity, organ predilection, and response to antifungal treatment. Owing to the relatively small discriminatory value of morphological features, yeast identification is based on biochemical tests which are available as a commercial kits (e.g., API 20C, ID 32C, BioMériéux) or automated devices (e.g., VITEK, BioMériéux). Special methods for identification of *C. albicans* and some other frequent *Candida* species have been utilized in mycological laboratories such as germ tube test, induction of chlamydospore formation on rice agar, or cultivation on chromogenic media (Pincus 2007). Methods for mould identification often rely on morphological description of a fungus based on colony appearance and microscopic picture. They are time-consuming and require experienced laboratory workers.

Apart from conventional techniques, more precise and rapid identification of yeasts as well as moulds is enabled by molecular analysis. Nucleic acid-based methods, including sequencing, and the establishment of protein profile by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) can be used now (see section 1.2.2.1).

1.1.3 Antifungal susceptibility testing

Standardized methods for antifungal susceptibility testing of yeasts and moulds approved by Clinical and Laboratory Standards Institute (CLSI) and The

European Committee on Antimicrobial Susceptibility Testing (EUCAST) are available in the formats of agar diffusion and broth dilution (Lass-Flörl et al. 2010). Interpretative breakpoints have been developed for almost all clinically relevant systemic antifungal drugs (Pfaller et al. 2011a,b). Even when prediction of clinical outcome on the basis of laboratory data has not yet been optimal, these breakpoints represent a considerable improvement in the rational management of IFDs (Lass-Flörl et al. 2011; Pfaller 2012). Present effort has been focusing on more precise species-dependent definition of these interpretative criteria for individual antifungals (Pfaller et al. 2010; Rambach et al. 2011).

1.1.4 Imaging methods

Imaging methods play an important role in the diagnosis of IFDs. The radiographic features of IA are often non-specific and appear late in the course of disease. High-resolution computed tomography and magnetic resonance imaging scans make it possible to detect both pulmonary and extrapulmonary IA and other IFDs with a relatively high degree of probability, but they require the existence of macroscopic lesions, which are usually markers of poor prognosis (Cuenca-Estrella et al. 2011).

Despite the relatively poor reliability of the methods indicated above, the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC/IFICG), the National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) recommend the indicated conventional diagnostic tests as gold standard for the definitions of diagnostic criteria characterizing IFD (De Pauw et al. 2008).

1.2 Culture-independent diagnostic tests

Among the many challenges in dealing with IFDs, early diagnosis is of paramount importance. The negative impact of delay in the initiation of adequate antifungal therapy on the outcome of patients with IFDs has become evident (Michallet and Ito 2009; Marr 2010). There have been great efforts therefore to develop new surrogate markers for diagnosis of IFD including improved non-culture methods such as non-invasive, more sensitive and faster techniques for detection of fungal cell components or DNA in peripheral blood and other body tissue and fluids.

1.2.1 Detection of fungal antigens

1.2.1.1 *Aspergillus* galactomannan

Galactomannan (GM) is a polysaccharide component of the cell wall of *Aspergillus* spp. that is released by the fungus into the blood stream during hyphal growth. Detection and quantification of this fungal component is considered to be a test specific for the diagnosis of IA (De Pauw et al. 2008). However, other filamentous fungi such as *Penicillium* spp. and *Paecilomyces* spp. contain lower, yet readily detectable GM amounts in their cell wall, thus leading to false-positive test results with regard to IA (Cuenca-Estrella et al. 2011). The GM sandwich Enzyme-linked immunosorbent assay (ELISA) (Platelia Aspergillus EIA, BioRad, USA) is the most commonly used commercially available test system approved for clinical screening by the Food and Drug Administration (FDA). Antigen determination by this test can be performed in serum, plasma, bronchoalveolar lavage fluid, cerebrospinal fluid and other body liquids. The sensitivity and specificity of the GM assay in range of 80% to 100% for IA varies significantly depending on the patient group, host factors, nutrition of the patient, administration of certain drugs and, most importantly,

on the threshold for GM positivity used (Wheat 2007; Einsele and Loeffler 2008). Based on practical experience and clinical study results, the cut-off value of galactomannan index was lowered from 1.5 (original manufacturer recommendation) to 0.5 for hemato-oncological patients, which has been associated with adequate sensitivity and specificity (Leeflang et al. 2008; Maertens et al. 2007; Meersseman et al. 2008). However, this issue is still controversial, and other cut-off values have been advocated (Herbrecht et al. 2002; Lai et al. 2007). The GM assay provides best results in hematologic malignancies and hematopoietic stem cells transplant recipients usually with good sensitivity and specificity, but the test has appeared less useful in children, solid organ transplant recipients and other immunocompromised patients (Herbrecht et al. 2002; Pappas 2010).

One of the most important limitations of the Platelia GM assay is the occurrence of false-positive results described in patients receiving certain β-lactam antibiotics such as piperacillin-tazobactam (Viscoli et al. 2004) or amoxicillin-clavulanate (Zandijk et al. 2008). Other possible sources of false-positive results are immunosuppressive drugs, namely cyclophosphamide (Cuenca-Estrella et al. 2011) or intravenous Plasma-Lyte® electrolyte solutions (Racil et al. 2007), dietary GM in pasta, cereals and formula milk, and cross reactivity with lipoteichoic acid of *Bifidobacteria* of the infantile gut microbiota (Dornbusch et al. 2009).

1.2.1.2 *Candida* mannan and anti-mannan antibodies

Mannan is released during IC and it can be detected by immunoassay tests with variable sensitivity (25%-85%) and a relatively satisfactory specifity (>80%). Serial samples are needed to overcome problems with rapid clearance of circulating mannan from sera and with the formation of antigen-antibody complexes. To get optimal diagnostic results the combination of tests for detection of mannan and anti-mannan antibodies should be considered (Prella et al. 2005; Alam et al. 2007). Alternative to *Candida* antigen and/or

antibody is the establishment of metabolic products of the yeast such as enolase or D-arabinitol/creatine ratio (Walsh et al. 1991; Walsh et al. 1995; Mitsutake et al. 1996; Yeo et al. 2006). An additional possibility is the detection of β-1,3-D-glucan, another cell wall component present in many fungi including *Candida* spp. although it is not immunogenic (see section 1.2.1.4). Promising results were provided by a study which combined β-1,3-D-glucan detection with antibodies against *C. albicans* germ tubes with sensitivity and specificity approaching 100% (Pazas et al. 2006).

1.2.1.3 *Cryptococcus* capsular antigen

Detection of the capsular polysaccharide antigen of *Cryptococcus neoformans* has an important place in diagnosis of meningitis and disseminated infections caused by this encapsulated yeast. There are commercial tests targeted to capsular glucuronoxylomannan, the main component of the antigen. Latex agglutination-based tests provide modestly sensitive results. Immunoassays (e.g., ELISA) are more reliable, especially in HIV patients (Yeo and Wong 2002; Antinori et al. 2005).

1.2.1.4 Panfungal β-D-glucan

An additional FDA-approved antigenemia ELISA assay detects the polysaccharide 1,3-β-D-glucan (BDG) which is, in contrast to galactomannan, present in the cell wall of most pathogenic fungi including *Candida*, *Aspergillus*, *Fusarium*, *Trichosporon*, *Saccharomyces* and *Acremonium* (Alexander 2002), thereby permitting broader detection of fungal pathogens. Notable exceptions include Mucorales and *Cryptococcus* which contain very low quantities of BDG in their cell walls (Cuenca-Estrella et al. 2011). However, some studies have recently described the utility of the test even for the detection of cryptococcosis and zygomycosis (Obayashi et al. 2008; Koo et al. 2009). The most frequently

used commercial assays for BDG detection are the Fungitell® (Associates of Cape Cod, USA) and Fungitec (Seikagaku) and there is no considerable difference in the accuracy of test results for the diagnosis of IFDs in patients with hematological malignancies (Lamoth et al. 2012). Although BDG methods appear to detect a broader range of fungal pathogens causing IFDs, none of these tests provide the possibility to identify the species involved. BDG detection in general provides good sensitivity and specificity in the range of 70% - 100% and 75% to 90%, respectively, including also neonatal patient populations (Kohno et al. 1993; Odabasi et al. 2004; Ostrosky-Zeichner L et al. 2005; Pazos et al. 2005; Couto et al. 2007; Lu et al. 2011; Mackay et al. 2011). Diagnostic relevance can be improved with two consecutively positive test results (Odabasi et al. 2004; Lamoth et al. 2012; Onishi et al. 2012).

However, like GM testing, BDG detection is also influenced by a number of factors interfering with the results and their interpretation. False-positive results are related to the administration of certain antimicrobials such as cefepime, colistin, ertapenem, cefotaxime and amoxicillin clavulanate (Marty et al. 2006). Additionally, some bacteria (e.g. pneumococci, *Alcaligenes faecalis, Pseudomonas aeruginosa* etc.) and exposure to BDG-containing materials such as cellulose filters or membranes for hemodialysis as well as immunoglobulin, albumin and other blood products have been documented as a source of false-positive results of BDG tests (Pickering et al. 2005; Mennink-Kersten et al. 2006; Mennink-Kersten et al.2008). Despite these shortcomings of both BDG tests, the EORTC/MSG Consensus Group included the GM and BDG detection methods in the diagnostic criteria for IFDs on the grounds of their standardization, validation, and commercial availability (De Pauw et al. 2008).

1.2.2 Molecular testing

1.2.2.1 Proteome analysis

A novel promising approach to laboratory diagnosis of fungal infections is the employment of MALDI-TOF-MS technology facilitating the identification of potentially pathogenic microbes including fungi, based on the measurement of their characteristic protein expression patterns (Seng et al. 2010). An increasing number of papers demonstrate the potential of the method to accelerate the identification process and to broaden the spectrum of fungal species that can be identified (van Veen et al. 2010; De Carolis et al. 2011). However, this methodology can be influenced by cultivation conditions in some fungi. The utility of direct identification from the blood and other biological samples remains to be confirmed on large scale of fungal pathogens and types of specimens (Pfohler et al. 2009; Bille et al. 2011; Ferreira et al. 2011).

1.2.2.2 Genome analysis

Important limitations of available methods for the diagnosis of IFDs have led to the development of fungal nucleic acid detection techniques. Several hundred publications on the application of PCR for the detection of fungal DNA have appeared and provided a new diagnostic option displaying great potential. Major advantages of molecular methods include the high sensitivity and rapid availability of results, which permit timely onset of antifungal therapy.

1.2.2.2.1 PCR formats

PCR-based methods belong to the most frequently used approaches to molecular fungus analysis either permitting species-specific, genus-specific or broad-range (panfungal) fungus detection. Several molecular formats exist

for qualitative and/or quantitative detection and identification of fungi. Standard PCR (El-Mahallawy et al. 2006; Raad et al. 2002) and PCR-ELISA (Löffler et al. 1998; Florent et al. 2006) belong to the first approaches established for clinical application, followed by nested PCR systems (Skladny et al. 1999; Holmes et al. 1994) developed to improve the detection limit of the assay. At present, a number of different variations of RQ-PCR procedures are available, allowing quantification of fungal load and, depending on the setting of the assay, pathogen identification at the species level (Basková et al. 2007; Schabereiter-Gurtner et al. 2007). Moreover, special methods such as nucleic acid sequence-based amplification (NASBA) (Park et al. 2011), multiplex PCR followed by DNA microarray (Spiess et al. 2007), fragment size analysis of variable regions of the fungal genome (Landlinger et al. 2009a), DNA sequencing (Leaw et al. 2006), hybridization to specific capture probes bound to microbeads (Landlinger et al. 2009b) or pyrosequencing (Borman et al. 2010) have been developed to identify fungal pathogens in clinical samples.

1.2.2.2.2 Target gene selection

The growing spectrum of fungal pathogens involved in IFDs and the requirement of highly sensitive fungus detection necessitated the selection of appropriate target genes facilitating detection and/or identification of clinically relevant fungal pathogens at adequate sensitivity and specificity levels. A variety of single- or multi-copy genes were exploited to date for different molecular approaches.

a) Single copy genes

A number of PCR assays that target single-copy genes of yeasts, in particular *Candida* spp., have been tested. The genes including actin (Kan 1993), chitin synthase (Jordan 1994), heat shock protein 90 (Crampin and Matthews 1993) and gene for cytochrome P-450 lanosterol-14-alpha-demethylase (Buchman et

al. 1990) have been tested. Targeting the topoisomerase II gene permitted identification of four different *Candida* species and *A. fumigatus* (Kanbe et al. 2003). In *Aspergillus* spp., copperzinc superoxide dismutase (Cu-ZnSOD) is a well-known potential marker of for IA. A recombinant Cu-ZnSOD of *A. fumigatus* has been produced by PCR amplification using homologous primers encoding the N-terminal sequence and the C-terminal region of the Cu-ZnSOD cDNA. The protein has been exploited as a diagnostic tool only to develop Western blot and ELISA assays for the detection of antibody responses in patients with IA. The option to target this protein in a PCR assay is worthy of consideration (Heldom et al, 2000).

There are limited data on PCR tests targeting single-copy genes for the detection of moulds other than *Aspergillus* spp. For the detection of *Paracoccidioides brasiliensis*, the gene encoding a 43 kDa glycoprotein (gp43) has been targeted (Bialek et al. 2000). *Pneumocystis jiroveci* was detected in BAL fluid targeting cell-division-cycle molecule 2 (Cdc2) (Arcenas et al. 2006).

b) Multi-copy genes

Ribosomal *DNA gene*. During recent years, the ribosomal DNA (rDNA) gene has been the most commonly targeted sequence offering the possibility to identify individual fungi (Landlinger et al. 2009a,b) or to detect a broad-range of fungal pathogens simultaneously (Löffler et al. 1998; Baskova et al. 2007; Schabereiter-Gurtner et al. 2007; Landlinger et al. 2010). The nuclear-encoded rDNA gene is a tandem array of at least 50-100 copies in the haploid genome of all fungi (Reiss et al. 1998). It exists as a multiple-copy gene family comprised of highly similar DNA sequences (typically 8-12 kb each) arranged in a head-to-toe manner. Each repeat unit is separated from the next by a non-transcribed region known as the intergenic spacer (IGS) region (Hillis and Dixon 1991). In nearly all basidiomycetes and several ascomycetes, the IGS is divided by the 5S rRNA gene into IGS1 and IGS2 (Fig. 1, Drouin and de Sá 1995). In most filamentous fungi and most other eukaryotes, the IGS is not

interrupted, and the 5S rRNA gene is found elsewhere in the nuclear genome (Wolters and Erdmann 1989; Drouin and de Sá 1995). The small subunit (SSU) rRNA (18S) gene, the 5.8S gene and the large subunit (LSU) rRNA (28S) gene are transcribed as a single primary transcript which is subsequently cleaved during processing in the nucleus into three final molecules (Fig. 1). These three genes are separated by two internal transcribed spacers (ITS1 and ITS2) which contain signals for processing the primary transcript. Transcription of the 5S rRNA gene is distinct from the other three genes (Hillis and Dixon 1991).

The coding regions of 18S, 5.8S and 28S nuclear rRNA genes evolved slowly, and are relatively conserved among fungi (White et al. 1990). The 18S region is about 1800 bp in size with both conserved and variable domain sequences. Sequence variations within this region have been used to assess the taxonomic relationship of the major groups of living organisms and to separate genera and species based on sequence polymorphisms (Einsele et al. 1997). However, the drawback in using this region for the identification of species is the relative sequence homology among species and the need to sequence a large number of bases for comparative analysis (Iwen et al. 2002).

The 5.8S region, on the other hand, is only about 160 bp long and highly conserved among most fungal organisms. Owing to the small size and the conserved sequence, it is not appropriate for phylogenetic studies to classify fungal species. However, this conserved sequence has been useful as a binding site for universal primers for the amplification of flanking spacer regions within the eukaryotic genome (White et al. 1990).

Finally, the 28S region, which is around 3400 bp in size in fungi, also contains both conserved and variable nucleotide sequences. In a study on RNAs from the large ribosomal subunit derived from eukaryotic cells, De Rijk (De Rijk et al 1996) reported that the length of sequences within the 28S region ranged from 2900 bp in yeast to over 5000 bp in *Homo sapiens*. Much of the 28S rRNA gene, however, is conserved among organism groups, thus limiting the usefulness of this region for species identification (Michot et al. 1984; Sandhu et al. 1995).

The ITS1 and ITS2 parts of the gene, which flank the 5.8S region, show extensive sequence diversity among major groups of eukaryotic organisms and even within species of the same organism group. Sequence sizes for each region vary from around 1000 bp in human cells to < 200 bp in some yeast (De Baere et al. 2002; Michot et al. 1984; Turenne et al. 1999). It was shown that these DNA spacers are also important for early transcription during rRNA processing (Iwen et al. 2002).

The sequence composition of the rDNA complex allows the design of universal primers, based on the conserved regions, which will amplify a certain fragment of the rDNA gene cluster in a large number of fungal species. Moroever, it permits the design of species-specific primers and probes, based on the variable regions, which can be used to identify individual species. Furthermore, the presence of multiple copies of the rDNA gene cluster in the fungal genome provides a basis for highly sensitive detection. Panfungal RQ-PCR approaches were established to permit rapid and highly sensitive detection of previously rare fungal pathogens such as Mucorales, hyalohyphomycetes and yeast-like fungi in addition to a broad spectrum of *Aspergillus* and *Candida* species (Landlinger et al. 2010; Vollmer et al. 2008).

Candida-secreted aspartic proteinase gene. The family of *Candida*-secreted aspartic proteinase genes (*SAP*s), which comprises at least nine related members in the *C. albicans* genome (De Bernardis et al 2001), are additional multi-copy genes that have been used as a target for the detection of *C. albicans* by PCR. Any number of unique primers can be designed to target specific homologous regions of the *SAP* genes (Bautista-Munoz et al. 2003; Flahaut et al. 1998).

Candida mitochondrial EO3 fragment. A 2- kb DNA fragment, EO3 present in multiple copies in the *C. albicans* genome was isolated for use in developing a detection method for serotypes A and B of *C. albicans* (Miyakawa et al. 1992).

Aspergillus mitochondrial b gene. The *Aspergillus* mitochondrial gene is an alternative multi-copy gene that may be targeted in PCR assays designed to detect *Aspergillus* spp. (Bretagne et al. 1995; Jones et al. 1998).

Aspergillus alkaline proteinase gene. The family of alkaline proteinase enzymes in *A. fumigatus* is known to be responsible for most if not all of the extracellular elastinolytic activity of the organism (Kothary et al. 1984). Isolation and sequence analysis of fragments from the alkaline proteinase (*ALP*) gene in different *Aspergillus* species have allowed the design of complementary oligonucleotide primers that have provided useful diagnostic PCR tests (Urata et al. 1997).

1.2.2.2.3 Technical aspects of fungal PCR

A number of issues that arose in the studies performed could potentially affect the clinical applicability of PCR-based testing. These include technical aspects such as the selection of appropriate clinical specimens, including tissue samples, the required sample size, the efficacy and safety of DNA extraction procedures, the availability of carefully optimized and standardized amplification assays, the validation of the techniques in large clinical trials and, very importantly, the correct interpretation of results (Preuner and Lion 2009).

a) Clinical samples

A variety of clinical specimens have been employed for the detection of IFDs including whole blood, serum, plasma, BAL, cerebrospinal fluid, and fresh or paraffin-embedded tissue biopsies from affected sites. However, the occurrence of bleeding complications in immunocompromised individuals with severe thrombocytopenia is a serious impediment to sample collection

requiring invasive procedures. The most suitable blood fraction for molecular fungus detection has been a matter of debate.

b) DNA extraction

Investigation of plasma or serum specimens, in which free-circulating DNA is present, may require less complex and laborious extraction protocols. In contrast, the inclusion of additional steps was shown to be a prerequisite for effective isolation of cell-associated fungal DNA. The extraction protocols therefore often include a mechanic disruption step with glass beads (Fredricks et al. 2005), an enzymatic pre-treatment step using recombinant lyticase (Löffler et al. 1998) or the combination of both procedures (Baskova et al. 2007) to effectively release fungal DNA. Liquid clinical samples used for PCR analysis vary greatly between 200 µl and 10 ml (Mengoli et al. 2009), where this fact may remain a particular problem of sufficient sample size in infants (Dornbusch et al. 2010). The fungal load in peripheral blood is mostly very low, ranging from less than 10 CFU/ml to a maximum of several hundred CFU/ml, a level rarely observed even in patients with proven fungal infection (Loeffler et al. 2002). These observations suggest that DNA extraction protocols that permit processing of larger initial samples volumes should be preferred. A plethora of different procedures for subsequent fungal DNA purification has been developed for manual or semi-automated applications showing comparable results. Some methods, particularly approaches including the use of spin-columns, appear to be more prone to carry-over contamination (White et al. 2006a).

c) False positivity

Contamination leading to false-positive results remains a major concern in PCR-based assays. It may occur at diverse stages of sample processing, including the collection of patient material and several steps of specimen handling in

the laboratory. The main source of contamination caused by airborne fungal spores can be largely avoided by sample collection under aseptic conditions and the use of laminar air-flow units for sample handling in the laboratory. Moreover, utilization of closed extraction formats e.g. the MagnaPure system (Roche Diagnostics, Penzberg, Germany) may further contribute to reducing the risk of contamination (Basková et al. 2007). The observation of contamination with fungal spores or traces of fungal DNA in a variety of commercially available reagents and consumables implies the need for a number of safety measures. These include sterile filtration of all solutions for efficient removal of fungal spores, careful testing of all new reagents and materials for the presence of fungal DNA and inclusion of multiple negative controls.

Beside the generation of false-positive results due to contamination, selection of an inappropriate detection system may be associated with other potential pitfalls. Screening methods for a broad range of fungal pathogens, which are preferred by the great majority of current molecular detection assays, exploit homologous regions within the fungal rDNA gene complex, as outlined above. Since these genes share significant homology with corresponding human genes, careful selection of PCR amplification and detection oligonucleotides is crucial to avoid co-amplification of human DNA and generation of false positive results (White et al. 2006).

Table 1.1. Laboratory methods in diagnostics of invasive fungal diseases

Method	Example	Main use
Convential		
Microscopy		
Wet preparation	KOH, saline	detection, mould identification
Stained preparation	Gram, Giemse, India ink	detection, mould identification
Flurescent dye staining	Calcofluor	detection (increased sensitivity)
Culture		
Routine media	Sabouraud	isolation, cultivation
Special media	Chromogenic agars	isolation, species identification (main species)
	Rice agar, Corn-Meal agar, Czapek-Dox agar	identification (morphological features)
	Mueller-Hinton, RPMI 1640	antifungal susceptibility testing
Histopathological		
Histological staining	methenamine silver stain, haematoxylin-eosin, mucicarmin, Papanicolau	detection (evidence of invasion)
Immunohistochemical	anti-*Aspergillus*	genus/species-specific detection
Immnunofluorescence	anti-*Candida*, anti-*Aspergillus*	genus/species-specific detection
Immunobiochemical		
Immunological		
Ab detection	anti-*Candida*, anti-*Aspergillus*	genus/species-specific detection
Ag detection	mannan, galactomannan, *Cryptococcus* antigen	genus/species-specific detection
Biochemical		
Fungal metabolites	enolase, arabinitol/creatinine	genus/species-specific detection
Fungal cell components	1,3-beta-D-glucan	detection
Molecular		
Nucleic-acid based		
Amplification-based	nested PCR, real-time PCR, microarray	detection, identification, strain typing
Sequencing-based	sequencing (Sanger), pyrosequencing	detection, identification, strain typing
Protein-based	MALDI-TOF-MS	identification, detection*, strain typing*

* to be confirmed and specified

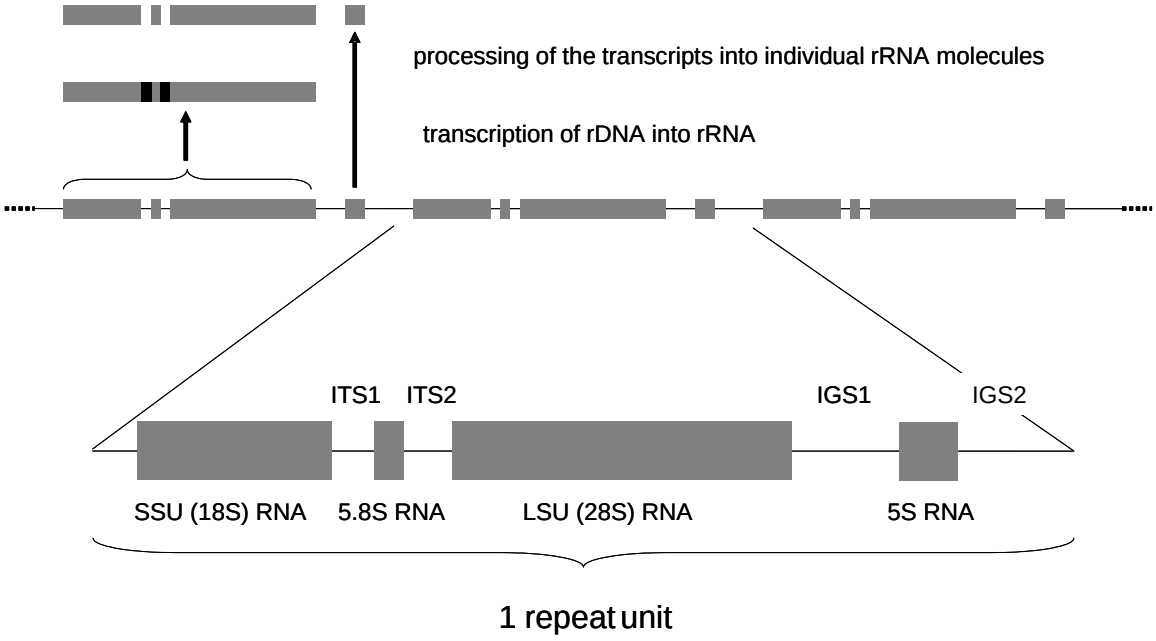


Figure 1.1. Organization of the nuclear-encoded ribosomal DNA genes (rDNA) of fungi. (Drouin and De Sá 1995) The genes exist as a multiple-copy gene family comprised of highly similar DNA sequences, present in up to several hundred copies. Each repeat unit (typicaly 8-12 kb each) has coding regions for one major transcript (containing the primary rRNA for a single ribosome), interrupted by one or two intergenic spacer (IGS) regions depending on the presence of the 5S rRNA gene.

SSU - small subunit, LSU - large subunit, ITS – internal transcribed spacer, IGS – intergenic spacer

2. STUDIES

2.1 The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of *Aspergillus* and *Candida* species

2.1.1 AIM OF THE STUDY

In the present study, we aimed at developing a rapid, sensitive and specific diagnostic test for the detection and accurate quantification of the most common pathogenic *Candida* and *Aspergillus* species. The design of the RQ-PCR assay should minimize the effect of point mutations on the detectability of the fungal species. No less importantly, the method should provide an economic approach to the screening and monitoring of invasive candidiasis and aspergillosis applicable to routine clinical diagnosis. This aspect was assessed by investigating a series of clinical specimens from patients with documented fungal infections.

2.1.2 MATERIALS AND METHODS

2.1.2.1 Fungal strains, bacteria and virus isolates

Fungal strains for PCR testing were obtained from the American Type Culture Collection (ATCC, Rockville, USA) and from the German Collection of Microorganisms (DSM, Braunschweig, Germany): *Aspergillus fumigatus* (ATCC 36607), *Aspergillus niger* (ATCC 10535), *Candida albicans* (ATCC 14053), *Candida dubliniensis* (ATCC MYA-646), *Candida glabrata* (ATCC 2001), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750), and *Aspergillus flavus* (DSM 818), *Aspergillus nidulans* (DSM 820), *Aspergillus terreus* (DSM 826), *Aspergillus versicolor* (DSM 1943),

Candida guilliermondii (DSM 70051), *Candida kefyr* (DSM 70073) and *Candida lusitaniae* (DSM 70102).

Prior to DNA extraction, the fungal isolates were cultured on Sabouraud dextrose agar at 30 °C; *Candida* isolates were cultured for 48 h and *Aspergillus* isolates for 72 h. Suspensions of all fungi (*Candida* species, cell suspension; *Aspergillus* species, conidia suspension) were prepared using an aliquot of cultured fungus resuspended in sterile 0.9% NaCl solution.

In addition, a panel of bacterial and viral microorganisms were selected for testing of cross-reactivity. The panel included *Enterobacter aerogenes*, *Eschericha coli*, *Haemophillus influenzae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus vulgaris*, human adenoviruses, Epstein–Barr virus, cytomegalovirus, varicella-zoster virus, herpes simplex virus types 1 and 2, and parvovirus B19.

2.1.2.2 Clinical materials

The clinical specimens from patients with documented fungus infections were obtained upon informed consent. The specimens included biopsies of pulmonary infiltrations (n=2) and peripheral blood (n=3) from haemato-oncological patients, bronchotracheal secretions (n=11) from intensive care patients, and a cornea control specimen with a culture-documented infection by *Fusarium solanii*, which is outside the detection spectrum of the Pan-AC assay (kindly provided by St Anna Children's Hospital, Vienna, Austria and the Institute of Hygiene and Medical Microbiology (IHMM) (Medical University of Vienna, Austria). Peripheral blood specimens from healthy volunteer donors were used to test for cross-reactivity with human DNA.

2.1.2.3 DNA extraction

All steps were performed in a laminar flow hood using one-way sterile utensils. Reagents used for DNA extraction were filtered through 0.2 µm sterile filters (Corning®, Corning Inc., Germany).

a) Fungal strains

A colony of each fungus culture was homogenized in 500 µl lyticase lysis buffer (LLB) [50 mM Tris (pH 7.6), 1 mM EDTA (pH 8.0), 0.2% 2-mercaptoethanol, 10 U/ml recombinant lyticase (Sigma, Steinheim, Germany)] and incubated at 37 °C for 2 h. After incubation, acid-washed glass beads 710–1180 µm in diameter (Sigma) were added and vortexed thoroughly for 2 min. A total of 400 µl supernatant were used for DNA extraction on a MagNA Pure compact instrument using the MagNA Pure compact nucleic acid isolation kit I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. The DNA was eluted in a total volume of 100 µl elution buffer (Roche Diagnostics). DNA concentrations were determined using the PicoGreen® dsDNA quantification kit (Molecular Probes Inc., Eugene, OR, USA) on the fluorescence spectrophotometer F-2500 (Hitachi, Japan).

b) Peripheral blood specimens

After hypotonic lysis of the erythrocytes from 3 ml blood using red cells lysis buffer [10 mM Tris (pH 7.6), 5 mM $MgCl_2$, 10mM NaCl (all Sigma)], as described by Loffler et al. (1997), the leukocytes were pelleted and resuspended in 470 µl LLB. The subsequent procedure followed the extraction protocol described above.

c) Respiratory secretions

Samples were centrifuged at 5000 g for 10 min. The supernatant was removed and 430 µl LLB was added. The extraction was performed as described above.

d) Solid specimens (e. g. lung biopsies and ocular specimens)

Solid material was mechanically disrupted and homogenized in 1x PBS. The samples were centrifuged at 5000 g for 10 min, the supernatant was removed and 430 µl LLB was added. The subsequent steps were as described above.

e) Bacteria and viruses

For the isolation of DNA from cultured bacteria and virus stocks, a commercially available kit (QIAamp DNA mini kit; Qiagen GmbH, Hilden, Germany) was used as recommended by the manufacturer.

2.1.2.4 Target sequence analysis and primer/probe design

Conserved nucleotide sequences of the fungal ribosomal multi-copy genes (18S, 5.8S and 28S) of clinically relevant *Candida* and *Aspergillus* species were selected and aligned using the BLAST search software, freely accessible at http://www.ncbi.nlm.nih.gov/BLAST/. Within the 28S large ribosomal subunit [between nucleotides 178 and 277 based on the sequence of the 28S rDNA gene of *C. albicans* (NCBI accession no. Z48339)], a highly conserved region was identified that spans less than 150 bp in length, thereby optimally fitting the requirements of RQ-PCR analysis using a hydrolysis TaqMan probe. Sequences for the forward primer, the reverse primer, and the universal probe (Table 2.1.2) were selected using Primer Express software (version 2.0; Applied

Biosystems) following the manufacturer's guidelines (Table 2.1.3). The probe was labelled with FAM (6-carboxyfluorescein) as a reporter molecule at the 5´-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher molecule at the 3´-end (Applied Biosystems). The optimal concentrations for the primers and the probe were assessed by serial analyses both from the functional and economic perspective, and were specified at 400 and 200 nM, respectively.

2.1.2.5 Real-time PCR

PCR reactions were set up in a total volume of 25 µl containing 12.5 µl universal master mix [2x concentration, including ROX-reference dye and uracil N´-glycosylase (UNG)] [Applied Biosystems, (AB)], 1% formamide, a mixture of the forward and the reverse primer (400 nM each), 200 nM Pan-AC hydrolysis probe, and 5 µl genomic DNA (gDNA). The mixture was transferred to 96-well optical microtitre plates (AB). Amplification was performed on the ABI 7700 sequence detection system (AB) using the following cycling parameters: 2 min at 50 °C (degradation of potentially present contaminating dUTP-containing amplicons by UNG), 10 min at 95 °C (inactivation of UNG and activation of AmpliTaq Gold DNA polymerase), followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C for target amplification. For absolute quantification of the fungal DNA, standard curves were prepared using serial logarithmic dilutions covering a range of five logs (10 fg–100 pg) gDNA derived from *C. albicans* and *A. fumigatus* as positive control samples.

2.1.2.6 Assessment of the detection limit

To determine the detection limit of fungal pathogens in peripheral blood, 1 ml EDTA-anticoagulated whole blood from healthy volunteer donors was spiked with tenfold serial dilutions of *A. fumigatus* conidia and *C. albicans* cells

(10^5 to 10^0 cells). The number of fungal particles was determined microscopically using a counting chamber. DNA extraction was performed according to the protocol outlined above (see section 2.1.2.3 b).

2.1.2.7 Controls

A number of precautions were undertaken to control the occurrence of false-positive results. Multiple no-template and non-homologous template controls were processed together with the specimens tested, as described previously (Watzinger et al. 2004). To document the efficiency of DNA extraction and amplification, various positive controls were included, as already published (Lion 2001). In clinical samples containing human cells, a single-copy housekeeping gene (β2-microglobulin) was co-amplified in parallel with the fungal targets (Lion 2001). For largely cell-free human specimens, defined concentrations of a seal herpes virus were spiked into the sample prior to DNA extraction and analysed by RQ-PCR to control for the potential occurrence of inhibitory effects (Watzinger et al. 2004).

2.1.3 RESULTS

2.1.3.1 Specificity of the Pan-AC assay

The ability of the Pan-AC system to detect all fungus species of interest (Table 2.1.1) was determined by testing DNA derived from cultures of reference strains, including *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. kefyr*, *C. lipolytica*, *C. lusitaniae*, *C. dubliniensis*, *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. versicolor* and *A. nidulans*. Target sequences of all species were amplified successfully and showed nearly identical amplification efficiencies (data not shown). Based on the sequence alignment of additional, less common *Aspergillus* and *Candida* species, the Pan-AC

RQ-PCR assay can be expected to cover several other members of these two genera with similar sensitivity.

The Pan-AC primers and probe (Table 2.1.2) were evaluated for possible cross-reactivity with bacterial or viral pathogens, both by sequence alignment using the BLAST software, and experimentally by testing the Pan-AC assay against bacterial and viral DNA from different organisms (see Methods). No crossreactivity between the Pan-AC primer/probe detection system and non-fungal pathogens was observed (data not shown). As indicated in Figure 2.1.1, DNA sequences from different human genes show significant homology with the fungal DNA sequence targeted by the Pan-AC assay. Analysing human DNA extracted from peripheral blood of healthy volunteer donors initially revealed some degree of crossreactivity. Different chemicals, including DMSO, glycerol and formamide, were tested in different concentrations (1–5%) to increase the stringency of the PCR reaction without simultaneously compromising the overall amplification efficiency. DMSO and glycerol completely inhibited the PCR reaction even at low concentrations (data not shown). By contrast, formamide concentrations of 1% abrogated the cross-reactivity with human DNA, while maintaining the high amplification efficiency of the PCR assay (Table 2.1.4). This concentration of formamide was, therefore, included both in the assessment of the detection limit and the testing of clinical specimens by the Pan-AC assay.

2.1.3.2 Detection limit of the assay

The detection limit of the Pan-AC assay was determined by testing serial dilutions of fungal gDNA derived from organisms representing yeasts (*C. albicans*) and moulds (*A. fumigatus*). The DNA concentration of the two fungal species was determined by fluorometric measurements, and serial logarithmic dilutions across a range of five logs (10 fg - 100 pg) were prepared. After PCR amplification, the cycle threshold (C_T) values of individual dilutions steps were plotted against the template amount, leading to typical standard

curves (Figure 2.1.2). The Y-intercept, which corresponds to the theoretical limit of detection, was determined at C_T 37.9 for *C. albicans* and C_T 38.9 for *A. fumigatus*. In view of the inter-assay variability of the technique in the range of ±1 C_T, this difference can be regarded as minor. The amplification efficiencies of the *Candida* and *Aspergillus* species listed in Table 2.1.1 revealed only marginal differences (data not shown). The standard curves presented are, therefore, applicable in quantitative analysis of all fungal species covered by the Pan-AC assay. The lowest template amount permitting accurate and reproducible quantification of fungal DNA was in the order of 10 fg.

In order to investigate the applicability of the Pan-AC assay to the analysis of clinical specimens, peripheral blood from healthy volunteer donors was spiked with tenfold serial dilutions of A*. fumigatus* conidia and *C. albicans* cells, covering a range between 10^5 to 10^0 organism/ml. The DNA was extracted and amplified as described in Methods. In patients with IFD, the fungal load in peripheral blood samples is generally low, often below 10 CFU/ml (Loeffler et al. 2000). Based on these observations, the sensitivity of the PCR assay is crucial for the detection and quantification of fungal pathogens. The detection limit of the Pan-AC assay in spiking experiments was less than 10 organisms per PCR reaction; however, for reproducible detection and quantitative analysis a higher volume of peripheral blood providing larger overall number of fungal organisms was beneficial (data not shown).

2.1.3.3 Analysis of clinical specimens

The Pan-AC assay was employed to investigate specimens from 17 haemato-oncological or intensive care patients with fungal infections diagnosed by other methodological approaches, including primarily culture techniques and DNA sequencing. According to the EORTC criteria, 4 patients were classified as having a possible IFD and 12 patients a probable IFD infection (Table 2.1.5, samples 1–16). Only for the patient from whom the cornea specimen was derived, was no EORTC classification available (Table 2.1.5, sample 17).

The Pan-AC assay revealed positive results in the clinical specimens studied, including lung biopsies of patients with pulmonary aspergillosis or candidiasis, peripheral blood specimens from patients with different types of candidaemia, and respiratory secretions positive for different *Aspergillus* species (Table 2.1.5). In one of the latter specimens, co-infection with *C. lusitaniae* was detected (Table 2.1.5, sample 13). The only clinical sample that tested negative by the Pan-AC assay was a cornea specimen, in which culture analysis revealed the presence of *F. solanii*. This specimen only served as a control because this fungus is not within the detection spectrum of the PCR assay presented (Table 2.1.5). Quantitative analysis of the fungal load in specimens positive by RQ-PCR was performed by using the appropriate standard curves, and the amount of fungal DNA determined was translated to the number of organisms on the basis of the estimated mean genome masses of *Aspergillus* and *Candida* species of 32 and 37 fg, respectively.

2.1.4 DISCUSSION

In the present report, a RQ-PCR assay is described permitting the detection and quantification of a broad range of clinically relevant *Aspergillus* and *Candida* species (Table 2.1.1) in a single reaction. A single primer pair and a universal probe were designed within a highly conserved region of the 28S large ribosomal subunit (Figure 2.1.1). The selected primer and probe sequences display a degenerated code (Table 2.1.2), which was a prerequisite for sensitive detection of fungal species differing from others at single nucleotide positions.

A major problem of fungal PCR assays is the high risk of contamination (Loeffler et al. 1999), which is attributable to the ubiquitous presence of airborne fungal spores, and traces of fungal DNA in a variety of reagents and other consumables. To avoid false-positive results, it is imperative therefore to control all materials used, both self-made and commercially available, for fungal contaminants, and to include multiple negative controls in each assay. The fungus detection assays must be performed under adequate experimental

conditions, which include the preparation of reagents and processing of clinical samples under a laminar flow biohazard hood. With appropriate precautions and controls, the RQ-PCR assay presented can serve as a reliable diagnostic tool for the detection and quantitative monitoring of pathogenic fungi in clinical specimens for the presence of IFD.

In patients testing positive, antifungal agents, such as voriconazole or caspofungins, could be used as first-line treatment, because these substances can be expected to cover the entire range of fungi detected by this assay. The use of agents with a narrower spectrum of antifungal activity, such as fluconazole or amphotericin B, would require the identification of the fungal species present, which can be performed by a number of molecular techniques, including, for example, the analysis of the variable ITS2 region or species-specific hybridization (Chen et al. 2000; Elie et al. 1998; Sandhu et al. 1995; Turenne et al. 1999).

In comparison to most earlier RQ-PCR approaches to the detection of invasive aspergillosis or candidiasis (Bu et al. 2005; Buchheidt et al., 2004; Kami et al., 2001; Kasai et al., 2006; Klingspor & Jalal, 2006; Spiess et al., 2003), the Pan-AC assay covers a considerably larger spectrum of pathogenic *Aspergillus* and *Candida* species in a single reaction, using a universal detection probe and a single primer pair. Our data indicate that the Pan-AC assay can be readily implemented in routine clinical diagnosis and monitoring of the majority of IFDs.

Table 2.1.1. GenBank accession numbers of fungal species used for 28S rDNA alignment and selection of sequences targeted by the Pan-AC assay.

Name	NCBI Acc. No.
Candida albicans	Z48339, X83717, L28817
Candida dubliniensis	U57685, AB031020
Candida glabrata	U44808, Z48341
Candida guilliermondii	AF374616, U45709
Candida kefyr	AF335978, Y15476
Candida krusei	U76347, Z48567
Candida lipolytica	AF335977
Candida lusitaniae	U44817
Candida parapsilosis	AF374609, Z48343
Candida tropicalis	AF267497, Z48346
Aspergillus flavus	AF027863, U28899
Aspergillus fumigatus	AF109335, U28460, Z48340
Aspergillus nidulans	AF109337, U29856
Aspergillus niger	U28815, AF109344
Aspergillus terreus	U28841, AF109340
Aspergillus vesicolor	AF433108, AF433059

Table 2.1.2. Primers and probe in the Pan-AC RQ-PCR assay.

Samples	Oligonucleotide sequences (5'-3')	Targets	NCBI Acc. No.	Nucleotide positions	Conc. (nM)
Fw primer	TGGGTGGTAAATTYCATCTAAAGCTA	*C. albicans*	Z48339	185-210*	400
Rev primer	CAAGTKCTTTTCATCTTTCSWTCAC	*C. albicans*	Z48339	245-270*	400
Probe (rev)	ACTTGTKCGCTATCGGTCTCYSGCC	*C. albicans*	Z48339	217-241*	200

* Nucleotide positions correspond to the NCBI sequence Acc. no. Z48339

Table 2.1.3. TaqMan® Primer/Probe design using the Primer Express ™ software: guidelines and recommendations

	Probe	**Primers**
GC content	20-80%	
	the strand with more Cs than Gs should be selected	the five nucleotides at the 3’ end should have no more than two G and/or C bases
Clustering of identical bases	avoid stretches of identical nucleotides, particularly Gs; clusters of four or more Gs should be avoided	
Melting temperature (Tm)	in a single-probe assay 68-70°C for TaqMan® Probe	58-60°C
	avoid design of probes with a G residue at the 5’ end	design the primers as close as possible to the probe without overlaps

Most consistent results are obtained for amplicon size ranges from 50-150 bp

The primers should be chosen according to the probe.

G guanine, C cytosine

Table 2.1.4. Influence of formamide concentration on the threshold cycle (C_T) and the fluorescence intensity of the reporter (ΔRn). The effect was tested for different fungus DNA template concentrations of both *Candida* and *Aspergillus* representatives ranging from 1 pg to 10 fg. The RQ-PCR results shown represent the effect of formamide observed in the lowest fungal template concentration tested (10 fg DNA of *C.albicans*). Although the overall fluorescence intensity decreased with rising concentrations of formamide, the concentration at which any cross-reactivity with human DNA was abrogated (1%) generally revealed only slightly increased C_T values.

Formamide conc. [%]	**C_T value**	**ΔRn**
0	33.60	2.41
0.5	34.17	1.72
1	34.12	1.55
1.5	35.46	1.10
2	36.61	0.95
2.5	40.86	0.50

Table 2.1.5. Fungal species identified in clinical specimens

Samples	Source	Diagnosis of IFD*	Microbiological evidence	Sequencing	Pan-AC PCR	C_T	Fungal DNA (fg)+
1	blood	probable	*C.glabrata*	N.D.	positive	34.8	8
2	blood	possible	N.D.	*C.albicans*	positive	28.1	600
3	blood	possible	N.D.	*C.krusei*	positive	37.0	2
4	lung biopsy	possible	N.D.	*A.fumigatus*	positive	35.7	4
5	lung biopsy	possible	N.D.	*C.lipolytica*	positive	30.7	130
6	respiratory tract	probable	*A.fumigatus, A.niger*	N.D.	positive	27.2	1429
7	respiratory tract	probable	*A.fumigatus*	N.D.	positive	32.4	40
8	respiratory tract	probable	*A.fumigatus*	N.D.	positive	31.6	69
9	respiratory tract	probable	*A.fumigatus*	N.D.	positive	26.1	2885
10	respiratory tract	probable	*A.fumigatus*	N.D.	positive	29.8	246
11	respiratory tract	probable	*A.nidulans*	N.D.	positive	32.4	40
12	respiratory tract	probable	*A.fumigatus, A.flavus*	N.D.	positive	29.9	230
13	respiratory tract	probable	*A.fumigatus, C.lusitaniae*	N.D.	positive	35.7	4
14	respiratory tract	probable	*Aspergillus spp.*	N.D.	positive	33.5	19
15	respiratory tract	probable	*A.fumigatus*	N.D.	positive	32.5	40
16	respiratory tract	probable	*A.fumigatus*	N.D.	positive	31.5	78
17	cornea	—	*F.solanii*	N.D.	negative	—	—

ND Not determined

* The diagnosis of IFD was performed according to the EORTC criteria (http://www.doctorfungus.org/lecture/eortc_msg_rev06.htm)

+ The numbers indicate the determined amount of fungal DNA at the beginning of the PCR-reactions. To calculate the fungal load in the clinical specimen investigated, the quantity of fungal DNA in femtograms assessed by RQ-PCR analysis can be translated to the number of fungal organisms on the basis of the estimated genome mass (*Aspergillus* 32 fg, *Candida* 37 fg) and the appropriate dilution factor of the sample analyzed.

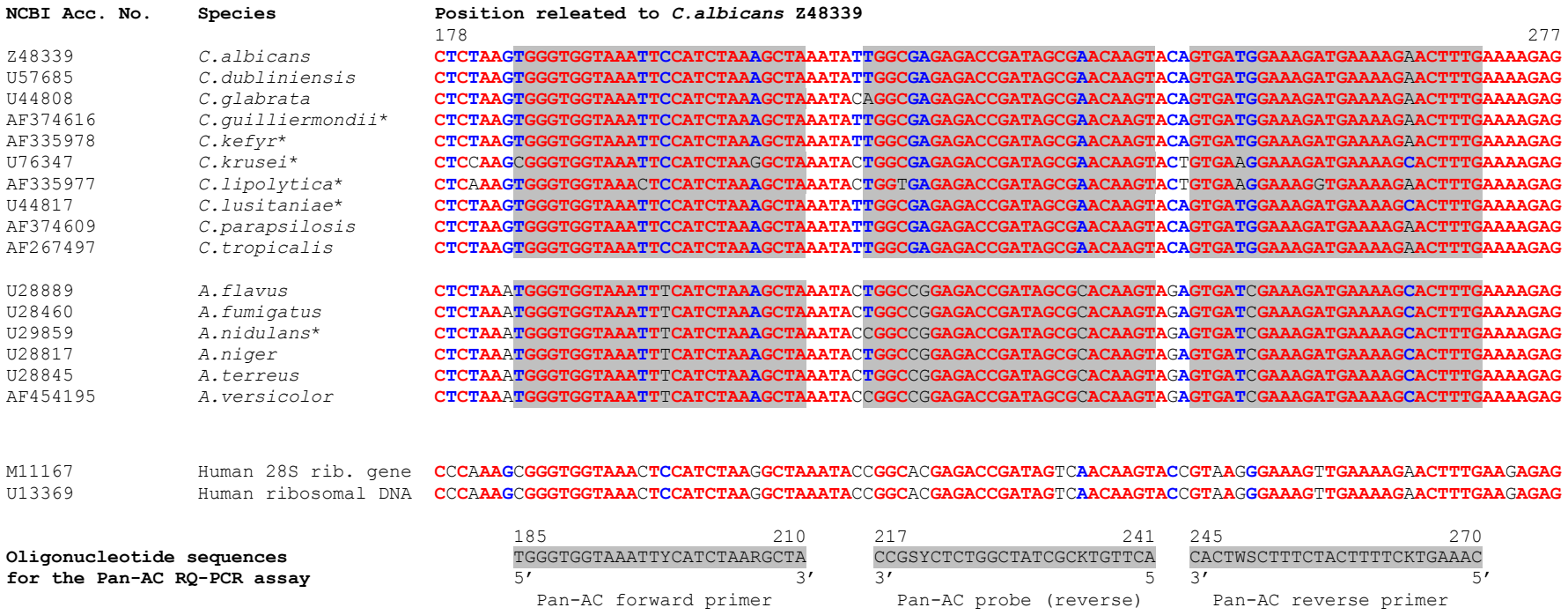


Figure 2.1.1. Multiple sequence alignment (Corpet F 1988) of a highly conserved region within the 28S rDNA gene of ten *Candida* species, six *Aspergillus* species and the corresponding region from the human genome. The primer pair and the probe (grey boxes) for the Pan-AC RQ-PCR assay were placed within this region. The indicated nucleotide positions refer to the sequece of *C.albicans* (NCBI accession no. Z48339). The asterisks indicate species that are listed under other names in the NCBI database: *C. guilliermondii = NCBI Pichia guilliermondii, C. kefyr = NCBI Kluyveromyces marxianus, C. krusei = NCBI Issatschenkia orientalis, C. lipolytica = NCBI Yarrowia lipolytica, C. lusitaniae = NCBI Clavispora lusitaniae, A. nidulans = NCBI Emericella nidulans.*

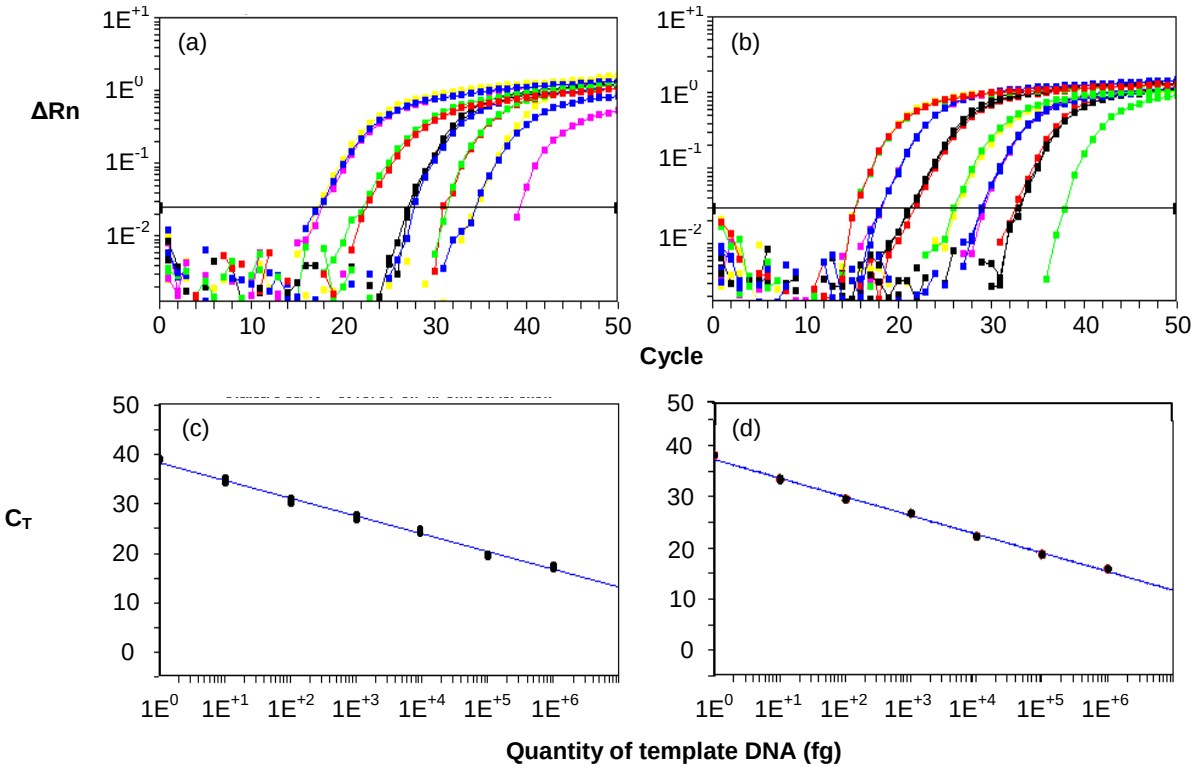


Figure 2.1.2. Amplification plots (a, b) and standard curves (c, d) based on serial dilutions of fungal gDNA ranging from 10 fg to 100 pg, derived from representatives of yeasts (*C. albicans*) (a, c) and moulds (*A. fumigatus*) (b, d). The assays underlying the generation of standard curves were performed in duplicates.

2.2 Diagnosis of invasive fungal diseases by a real-time panfungal PCR assay in immunocompromised patients

2.2.1 AIM OF THE STUDY

IFD is a life-threatening event in immunocompromised patients, and there is urgent need for reliable screening methods facilitating rapid and reliable broad detection of pathogenic fungi. To address this task, we aimed at establishing a RQ-PCR assay for detection of a wide spectrum of fungal pathogens including fungal species of both common and emerging fungal genera. The potential clinical utility of the assay was assessed in a combined prospective and retrospective study in severely immunocompromised pediatric patients displaying a high risk of IFD.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Fungal strains, bacteria, and viral isolates

Fungal strains for PCR testing (Table 2.2.1) were obtained from different institutions including the ATCC, (Rockville, USA), the DSM, (Braunschweig, Germany), and the IHMM, (Medical University of Vienna, Austria).

2.2.2.2 Clinical materials

Clinical specimens from consecutive patients were obtained after informed consent, and were prospectively collected as specified below. In total, 618 peripheral blood specimens from 125 pediatric hemato-oncological patients undergoing intensive chemotherapy (n=65) or allogeneic stem cell transplantation (n=60) were analyzed during 150 episodes of febrile neutropenia.

Whenever possible, specimens were collected at first onset of fever, within 48 hours thereafter, and at subsequent time points in the course of the febrile episode, upon availability. On average, four peripheral blood samples were investigated during each episode (range 1-15). In selected instances, depending on availability, additional types of specimens derived from primarily sterile sites of suspected infection were collected and subjected to molecular analysis. These included cerebrospinal fluid (n=11) and lung biopsies (n=2) in patients with suspected CNS or pulmonary involvement, respectively, in order to correlate the data with PCR findings in peripheral blood samples. The specimens used as training set for initial data assessment were provided by the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands, and the specimens used as validation set by the St. Anna Children's Hospital of Vienna, Austria. Plasma or serum was usually collected from 3 ml samples of peripheral blood and all samples were stored at -80°C until further processing. Peripheral blood specimens from healthy volunteer donors were used to test for cross-reactivity with human DNA (see below).

2.2.2.3 DNA extraction

The isolation and purification of DNA from fungal strains, as well as of fungal, human, bacterial and viral DNA from different clinical materials was performed essentially as described earlier (Baskova et al. 2007).

2.2.2.4 Real-time PCR

The panfungal PCR detection assay included two separate reactions, I and II, each covering different subsets of fungal pathogens, as outlined in Table 2.2.1. Both PCR reactions targeted a highly conserved region of the 28S rRNA multicopy gene (Figure 2.2.1). The PCR reactions were set up in a total volume of 25 µl containing Gene Expression Mastermix 2x with UNG (AB, Foster City,

USA), primers and probes as listed in Table 2.2.2, and molecular biology grade water (Eppendorf, Hamburg, Germany). Locked nucleic acid (LNA) nucleotides were included in the primer and probe sequences to obtain high specificity despite short length (Tolstrup et al. 2003). The amplification was performed on a TaqMan 7900 instrument using the following standard protocol: 2 min at 50°C (UNG-mediated degradation of potentially present contaminating amplicons containing dUTP), 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C (Watzinger et al. 2004).

2.2.2.5 Assessment of cross-reactivity with bacterial, viral and human DNA

Potential cross-reactivity of the panfungal detection assay with non-fungal microorganisms was analyzed by testing a total of 20 different bacterial and viral pathogens. Multiple isolates of DNA derived from different human peripheral blood specimens were also tested for cross-reactivity in this setting (data not shown).

2.2.2.6 Controls

A number of positive and negative controls were included in the panfungal real-time assay as described earlier (Watzinger et al. 2004). In view of the lack of “fungus-grade” reagents guaranteed to be free of contamination by fungal particles or DNA, highly stringent measures to control and exclude contamination were performed to minimize the risk of false-positive results of the molecular screening assay.

2.2.2.7 Specificity of the real-time PCR assays

Differences in the detection efficiency of individual pathogens determined by testing defined concentrations of fungal species were in the range

of +/-1 C_T value. The only exceptions were the fungal species *Geotrichum candidum*, *Malassezia furfur*, *Mucor hiemalis, Candida lipolytica,* and *Acremonium strictum* which revealed higher C_T values (in the range of +3), apparently due to mismatches within regions targeted by the primers (Figure 2.2.1).

2.2.2.8 Sensitivity of the real-time PCR assays

To determine the detection limit of the real-time PCR assays, serial logarithmic dilutions across a six-log range (1 fg to 100 pg) of genomic DNA derived from *A. fumigatus* and *C. albicans,* as representatives for moulds and yeasts, respectively, were analyzed. Standard curves were generated on the basis of the amplification profile (Figure 2.2.2).

2.2.2.9 Definition of PCR positivity and DNAemia

All clinical specimens were analyzed in duplicates. A PCR result was regarded as positive when both C_T values were either ≤ 36.5 in reaction I or ≤ 37.5 in reaction II, reflecting the respective y-intercept values. In the event of single positive values of the duplicate measurement, the C_T had to be ≤ 34.5 in reaction I and ≤ 35.5 in reaction II to be regarded as PCR-positive. For the diagnosis of fungal DNAemia, a minimum of two PCR-positive peripheral blood specimens derived at subsequent time points during close follow-up investigation were required. In the rare instances in which only one specimen from a febrile neutropenic episode was available, a single PCR-positive result was regarded as indicative of DNAemia.

2.2.2.10 Statistical analysis

Results of panfungal PCR analysis in relation to the presence or absence of possible, probable, or proven IFD by the EORTC criteria were used for the calculation of sensitivity and specificity, and the corresponding negative (NPV) and positive predictive values (PPV) of the assay. Individual values and the corresponding 95% confidence intervals (CI) were calculated using the method of Wilson (Altmann et al. 2000).

2.2.3 RESULTS

2.2.3.1 Design and principle of the panfungal PCR assay

The universal primers and probes for the two-reaction panfungal PCR assay were judiciously selected on the basis of their homology to highly conserved sequences within the 28S gene based on the sequence alignment of more than 80 fungal species (Tables 2.2.1 and 2.2.2). The primers display a degenerated code required for efficient amplification of fungal species that differ from others at single nucleotide positions. In order to obtain optimal binding affinity, LNA modifications of individual nucleotides were included in the hybridization probes and the reverse primers (Table 2.2.2). Owing to the exploitation of a multi-copy target gene, the detection limit of the panfungal PCR assay is in the range of 1 fg fungal DNA per reaction, which corresponds to a fraction of a single fungal genome equivalent (Figure 2.2.2).

2.2.3.2 Detection spectrum and specificity of the panfungal PCR assay

To document the detection spectrum of the two-reaction panfungal PCR assay, multiple strains/isolates of 61 different fungal species (Table 2.2.1), were analyzed experimentally in repeated tests. However, based on extensive

sequence alignments, the panfungal real-time PCR assay presented can be expected to cover more than 80 different fungal species (Table 2.2.1). Reaction I was designed to permit detection and quantitative assessment of a variety of moulds (n≥ 29), whereas reaction II targets a broad range of yeasts and Zygomycetes (n≥ 58). The assay therefore permits the detection of co-infections by different fungal genera, which may not be uncommon in immunocompromised individuals.

Cross-reactivity between reactions I and II was observed at a high concentration of fungal template DNA (≥ 100fg/reaction). However, the amplification efficiencies for moulds and yeasts/Zygomycetes by the appropriate detection systems were about 3 logs higher, thereby permitting clear identification of the dominant class of fungal pathogen present. At the concentrations of fungal pathogens observed in most clinical specimens of immunocompromised individuals, the cross-reactivity was not relevant. The lack of cross-reactivity of the panfungal PCR assay with non-fungal pathogens or human genomic DNA was assessed by extensive testing of various bacterial and viral DNA isolates, and peripheral blood specimens of healthy volunteer donors, as specified in the Materials and Methods section.

2.2.3.3 Analysis of clinical specimens by the panfungal PCR assay in relation to the EORTC criteria for IFD

The analyses of clinical specimens were performed in a double-blind fashion to prevent any bias or influence on treatment decisions. First, a training set of 165 peripheral blood specimens derived from 43 pediatric patients with high risk of IFD was analyzed by the panfungal PCR assay in a retrospective study in order to generate preliminary data on the potential clinical utility of the test system. The patients were generally in febrile neutropenia at the time of sample collection. In total, 71 specimens (43%) tested PCR-positive, 34 in reaction I, 15 in reaction II, and 22 in both reactions with C_T values clearly beyond the level of potential cross-reactivity. The results of molecular testing were evaluated

in relation to the criteria proposed by the EORTC (De Pauw et al. 2008), and indicated that all patients with proven or probable IFD, and all but one case with possible IFD tested repeatedly PCR-positive, as specified in Table 2.2.3. In three instances, both PCR reactions revealed positive results indicative of mixed infections (Table 2.2.3).

Five patients received empirical antifungal therapy on the basis of clinical suspicion of fungal infection, which did not meet the EORTC definition of possible IFD (generally by absence of an appropriate clinical criterion), and three of these patients were also positive by the panfungal PCR assay (Table 2.2.3). However, consecutive PCR-positive results were also obtained in 6 of 27 instances with no clinical indication of IFD in patients who were on antifungal prophylaxis only or did not receive any kind of antimycotic treatment (Table 2.2.3). Hence, analysis of the training data set indicated a good correlation between repeated positivity of the panfungal PCR assay and the presence of proven, probable, and possible IFD by the EORTC criteria, by revealing a sensitivity of the panfungal screening assay of 91% (95%CI: 62-98%), and a NPV of 95% (95%CI:78-99%). Although it might be more appropriate to calculate the NPV of the panfungal PCR assay on the basis of proven and probable IFD only, we have also included patients classified as having a possible IFD in the calculation in order to obtain a value applicable to any level of IFD according to the EORTC criteria.

A problem in the specificity of panfungal PCR-positive results became particularly apparent in the presence of single positive tests, and in follow-up samples collected at extended time intervals (>3 days) during febrile neutropenia. Based on the presence of positive PCR tests, according to the definition indicated in Materials and Methods, the specificity of the assay was 78% (95%CI:59-89%), and the PPV 63% (95%CI:39-82%).

On the basis of insights gained from analysis of the training set, a larger cohort of immunocompromised pediatric patients was investigated, with a focus on febrile neutropenic episodes and the requirement of consecutive samples collected at short time intervals (1-2 days). A total of 453 consecutive peripheral blood samples derived from 107 febrile neutropenic episodes in 82 pediatric

patients with hemato-oncological malignancies (n=22) or hematopoietic stem cell transplant recipients (n=60) were analyzed. Similar to the first cohort tested, 212 specimens (47%) tested PCR-positive, 122 in reaction I (58%), and 45 in reaction II (21%). Forty five samples (21%) were positive in both reactions, with differences in C_T values clearly beyond the level of potential cross-reactivity, hence indicating a mixed infection. Overall, 51 of 107 febrile neutropenic episodes investigated revealed repeatedly positive test results by the panfungal real-time PCR assay (Table 2.2.4). There was no statistically significant difference between allogeneic stem cell transplant recipients and non-transplant patients with regard to the occurrence of panfungal PCR-positivity in peripheral blood during the neutropenic febrile episodes (50% vs 39%; two-sided p-value = 0.29). PCR-positivity was observed in all cases of IFD according to EORTC criteria (n=16), revealing a sensitivity of 100% (95% CI: 81-100%) and an NPV of 100% (95% CI: 90%-100%).

All patients with proven, probable, and possible IFD (n=16) tested repeatedly PCR-positive, as specified in Table 2.2.4. In five instances, both PCR reactions revealed positive results indicative of mixed infections with different fungal genera (Table 2.2.4). In all 16 patients with IFD, positive PCR results were already detected in the specimens collected at first onset of fever within a febrile neutropenic episode.

In 48 instances, patients with febrile neutropenia received empirical antifungal treatment despite the lack of findings supporting the presence of IFD according to the EORTC criteria. Only 25 of these patients revealed evidence of IFD by panfungal PCR analysis (Table 2.2.4). In patients with no clinical indication of IFD, including individuals receiving antifungal prophylaxis only and individuals without any antimycotic treatment, repeatedly positive test results by the panfungal PCR assay were obtained in 10 of 43 (23%) febrile neutropenic episodes (Table 2.2.4). The observations made in the validation patient cohort therefore reflected a specificity of the assay at the level of 77% (95% CI:62-87%) and a PPV of 62% (95% CI:42%-76%).

Evaluation of the molecular assay in both patient cohorts combined revealed a sensitivity of 96% (95%CI:82-99%), and a specificity of 77% (95%CI:66-85%), corresponding to a NPV of 98% (95%CI:90-100%), and a PPV of 62% (95%CI:47-75%).

2.2.4 DISCUSSION

Over the last years, a small number of prospective studies have assessed the clinical utility of different PCR assays in hematopoietic stem cell transplantation recipients and/or patients with hematological malignancies (Jordanides et al 2005; Klingspor and Jalal 2006; Hebart et al. 2000; Boudewijns et al. 2006; Buchheidt et al. 2001). The reported sensitivity and the NPV were moderate to high. The specificity was generally lower, but could be significantly improved by establishing the requirement of two consecutive positive PCR results as a diagnostic criterion (Cuenca-Estrella et al. 2009; Mengoli et al. 2009). However, the diagnostic reliability of PCR assays in peripheral blood and serum specimens has been described to be rather limited (White et al. 2006a;b).

The panfungal real-time PCR assay presented was specifically adapted to highly sensitive detection of fungal pathogens in peripheral blood or serum. In the current combined prospective and retrospective study, more than 600 clinical specimens from 150 febrile neutropenic episodes in pediatric patients with high risk of invasive fungal disease were investigated by the panfungal real-time PCR assay described. Evaluation of the molecular screening data, which had been generated in a double-blind fashion, revealed an excellent correlation with the EORTC definitions of proven, probable, and possible invasive fungal disease. The sensitivity and the NPV in the validation cohort of patients were in the range of 100% indicating that molecular screening might be instrumental in preventing unnecessary treatment. The possible benefit of this notion is underlined by the observation that nearly half of the febrile neutropenic episodes studied were treated empirically by antifungal agents, despite the lack of evidence for IFD according to the EORTC criteria. The absence of fungemia

determined by the panfungal PCR assay in about 50% of these episodes suggests that a high proportion of patients were probably over-treated.

The limited specificity and PPV of the panfungal PCR assay reflect the fact that even in severely immunocompromised patient populations, repeated or persistent detection of fungemia by sensitive, broad-spectrum PCR methods may not generally indicate an imminent risk of severe fungal disease. This observation might be interpreted as frequent occurrence of false positivity, most likely attributable to contamination. However, owing to the processing of test materials in a sterile environment, the use of internally tested reagents and the number of controls included in each assay, it may be an explanation for some, but very likely not all seemingly false-positive findings. Of the PCR-positive patients who received either no antifungal treatment at all or prophylaxis only (n=16), fungal disease was diagnosed during the subsequent clinically documented febrile episode early thereafter in two patients, raising the possibility that subclinical fungemia was present at the time of the febrile episode investigated. In two additional patients, fungus-positive cultures from urine and throat were obtained at the time of PCR positivity, indicating the presence of fungal infection or colonization. However, based on the current EORTC criteria, these patients could not be classified as having IFD at any level of probability. In five patients, treatment with semi-synthetic penicillins was documented during the febrile episodes investigated. These substances were reported to yield false-positive results in serological fungus detection tests, and it cannot be excluded that the occurrence of cross-reactivity with molecular detection assays might have accounted for false positivity of the panfungal PCR assay. In seven patients, no findings possibly explaining the PCR-positive results could be identified.

The limited correlation between positive panfungal PCR results and imminent fungal disease may also reflect the relatively common occurrence of fungemia caused by environmental fungi, such as *Alternaria* or *Cladosporium* species, which might display low pathogenicity even in immunosuppressed individuals (Aimanianda et al. 2009; Pastor and Guarro 2008). To assess this

notion, clinical implementation of methods facilitating reliable identification of these species at an adequate level of sensitivity would be required. A number of methods for rapid typing of fungal pathogens have been published (Chen et al. 2000; De Baere et al. 2002; Landlinger et al. 2009a; Turenne et al. 1999; Das et al. 2006; Diaz and Fell 2004; Boyanton et al. 2008; Putignani et al. 2008; Campa et al. 2008; Spiess et al. 2007; Zeng et al. 2007), and the development of novel and expectedly more potent methods is currently underway, including also approaches based on the exploitation of nano-technological devices (Landlinger et al. 2009b). Rapid and sensitive techniques for the identification of putatively non- or low-pathogenic environmental fungal species that may cause clinically silent fungemia could improve the interpretation of results obtained by broad-spectrum PCR screening methods. This might contribute to increased specificity of molecular monitoring with regard to more reliable assessment of the expected clinical relevance of fungemia. Hence, routine clinical implementation of methods for rapid identification of fungal species could provide a basis for better assessment of the need for treatment and for selection of the most appropriate antimycotic agent, if therapy appears to be required.

The lack of a gold standard for the assessment of IFD presents a problem in the interpretation of new diagnostic approaches. The criteria of proven, probable, and possible IFD proposed by the EORTC are widely accepted, but the diagnostic tests permitting the assessment of proven or even probable infection, such as histological examination of targeted biopsies, image-guided analyses (e.g. computer tomography), blood cultures, and serological tests are not available in many high-risk patients including also a large proportion of patients in the present study. The reliability of serological testing by galactomannan in pediatric patients is, however, questionable. It has been reported that galactomannan tests frequently yield false-positive results in the pediatric population. This observation was mainly attributable to dietary factors [e.g. various brands of milk formulas (Gangneux et al. 2002; Steinbach 2005) or soybean protein (Murashige et al. 2005)] and, in the general population, to the use of semi-synthetic penicillins (Adam et al. 2004; Zandijk et al. 2008).

Routine employment of a broader set of diagnostic methods including image-guided analysis, blood culture, and serological testing in carefully designed clinical studies would be required to assess and finally exploit the full diagnostic potential of the panfungal PCR screening assay presented. The design of appropriate large-scale clinical studies is currently underway.

Table 2.2.1. Fungal species/isolates detected by the panfungal RQ-PCR assay

Reaction I	**Reaction II**
Experimetally tested	**Experimetally tested**
Acremonium strictum	*Absidia corymbifera*
Alternaria alternata	*Blastoschizomyces capitatus*
Aspergillus candidus	*Candida albicans*
Aspergillus clavatus	*Candida allociferrii*
Aspergillus flavus	*Candida colliculosa*
Aspergillus fumigatus	*Candida cylindracea*
Aspergillus glaucus	*Candida dubliniensis*
Aspergillus nidulans	*Candida famata*
Aspergillus niger	*Candida glabrata*
Aspergillus terreus	*Candida guilliermondii*
Aspergillus versicolor	*Candida inconspicua*
Fusarium oxysporum	*Candida kefyr*
Fusarium proliferatum	*Candida krusei*
Fusarium solani	*Candida lambica*
Fusarium verticillioides	*Candida lipolytica*
Penicillium chrysogenum	*Candida lusitaniae*
Penicillium citrinum	*Candida membranaefaciens*
Penicillium marneffei	*Candida norvegensis*
Penicillium purpurogenum	*Candida parapsilosis*
Penicillium simplicissmum	*Candida pelliculosa*
Scedosporium apiospermum	*Candida rugosa*
	Candida sake
Based on sequence alignments	*Candida tropicalis*
Aspergillus ochraceus	*Candida utilis*
Aspergillus penicillioides	*Candida zeylanoides*
Aspergillus ustus	*Cryptococcus albidus*
Bipolaris eleusines	*Cryptococcus laurentii*
Cladosporium cladosporioides	*Cryptococcus neoformans*
Cladosporium oxysporum	*Cunninghamella bertholletiae*
Histoplasma capsulatum	*Geotrichum candidum*
Scedosporium prolificans	*Malassezia furfur*

Table 2.2.1. (*Continued*)

Reaction I	Reaction II
	Malassezia sympodialis
	Mucor hiemalis
	Mucor mucedo
	Mucor racemosus
	Rhizopus oryzae
	Trichosporon asahii
	Trichosporon cutaneum
	Trichosporon inkin
	Saccharomyces cerevisiae
	Based on sequence alignments
	Apophysomyces elegans
	Basidiobolus ranarum
	Cokeromyces recurvatus
	Mucor circinelloides
	Mucor ramosissimus
	Mucor rouxii
	Rhizomucor variabilis
	Rhizopus azygosporus
	Rhizopus microsporus
	Rhizopus stolonifer
	Rhodotorula mucilaginosa
	Saksenaea vasiformis
	Trichosporon asteroides
	Trichosporon moniliiforme
	Trichosporon mucoides
	Trichosporon ovoides

Human pathogenic fungi covered by the panfungal PCR detection assay including reactions I and II.

Table 2.2.2. Primers and probes used in the panfungal real-time PCR-assay

Name	Primer/ Probe	Oligonucleotide Sequence [5'-3']	Conc. (nM)	Acc.No.[a]	Position
Reaction I	Fw I	TAAAGCTAAATAYYGGCCRGAGA	400	U28460	257-279
	Rev I	CT[T]TYCAAAGTGCTTTTCA[T]C	400	U28460	329-309
				Z48339	277-255
	Rev II	CTCT[T]TTCAAAGTTCTTTTCA[T]C	400	U28460	331-309
				Z48339	277-255
	Probe I	ACT[T]GT[G]CG[C]TA[T]CG	40	U28460	295-281
Reaction II	Fw I	GGGTGGTRARYTCCWTCTAARGCTAA	400	Z48339	186-211
	Fw II	GGGWGGTAAATCYCWCCTAAAGCTAA	400	Z48339	186-211
	Rev I	CTCT[T]TYCAAAGTKCTTTTCA[T]C	400	U284460	329-309
				Z48339	277-255
	Probe II	A[C]TT[G]T[T]C[G][C]TA[T]CG	50	Z48339	241-227

Primer and probe sequences included in the panfungal PCR assay. Reaction I covers moulds and reaction II primarily yeasts and Zygomycetes. Nucleotides within brackets carry LNA modifications (22).

[a] The sequence accession numbers are based on *A. fumigatus* (U28460) and *C. albicans* (Z48339).

Table 2.2.3. Analysis of clinical specimens: training set

	EORTC IFD-positive						EORTC IFD-negative[a]		
	Proven IFD		Probable IFD		Possible IFD		Empirical treatment	Antifungal prophylaxis	No antifungal prophylaxis/ treatment
Clinical assessment	Asp	Can	Asp	Can	Asp	Can			
Febrile episodes (n=43)	1	1	4		5		5	14	13
PCR reaction I positive (n=10)			3		3		1	2	1
PCR reaction II positive (n=2)		1							1
PCR reaction I and II positive (n=7)	1		1		1		2	1	1
PCR negative (n=24)					1		2	11	10

Abbreviations: Asp, *Aspergillus*, Can, *Candida*; EORTC, European Organization for Research and Treatment of Cancer

Correlation of PCR results with clinical findings according to the EORTC criteria of IFD in the training cohort including febrile episodes in 43 patients.

The cases of proven, probable and possible IFD revealed the presence of moulds (n=6; reaction I), yeasts/Zygomycetes (n=1; reaction II) or positivity in both reactions (n=3), respectively. Clinical assessment based on blood culture, HR-CT or histological analysis of targeted biopsies revealed findings indicative of *Candida* (Can) in one patient and *Aspergillus* (Asp) in 10 patients identified as having IFD according to the EORTC criteria.

[a] EORTC negative indicates the lack of evidence for possible, probable, or proven IFD according to the consensus criteria. For these cases, the therapeutic (empirical) and prophylactic employment of antifungal agents as well as the absence of any administration of antifungals are indicated.

Table 2.2.4. Analysis of clinical specimens: validation set

	EORTC IFD-positive						EORTC IFD-negative[a]		
	Proven IFD		Probable IFD		Possible IFD		Empirical treatment	Antifungal prophylaxis	No antifungal prophylaxis/ treatment
Clinical assessment	Asp	Can	Asp	Can	Asp	Can			
Febrile episodes (n=107)	3		1		10	2	48	11	32
PCR reaction I positive (n=29)	2		1		7		14	1	4
PCR reaction II positive (n=6)						1	2	1	2
PCR reaction I and II positive (n=16)	1				3	1	9	1	1
PCR negative (n=56)							23	8	25

Abbreviations: Asp, Aspergillus, Can, Candida; EORTC, European Organization for Research and Treatment of Cancer

Correlation of PCR results with clinical findings according to the EORTC criteria of IFD in the validation cohort including 107 febrile episodes in 82 patients.

The cases of proven, probable and possible IFD revealed the presence of moulds (n=10; reaction I), yeasts/Zygomycetes (n=1; reaction II) or positivity in both reactions (n=5), respectively. Clinical assessment based on blood culture, HR-CT or histological analysis of targeted biopsies revealed findings indicative of *Candida* (Can) in two patient and *Aspergillus* (Asp) in 14 patients identified as having IFD according to the EORTC criteria.

[a] EORTC negative indicates the lack of evidence for possible, probable, or proven IFD according to the consensus criteria. For these cases, the therapeutic (empirical) and prophylactic employment of antifungal agents as well as the absence of any administration of antifungals are indicated.

Panfungal detection reaction I

Based on experimental evidence

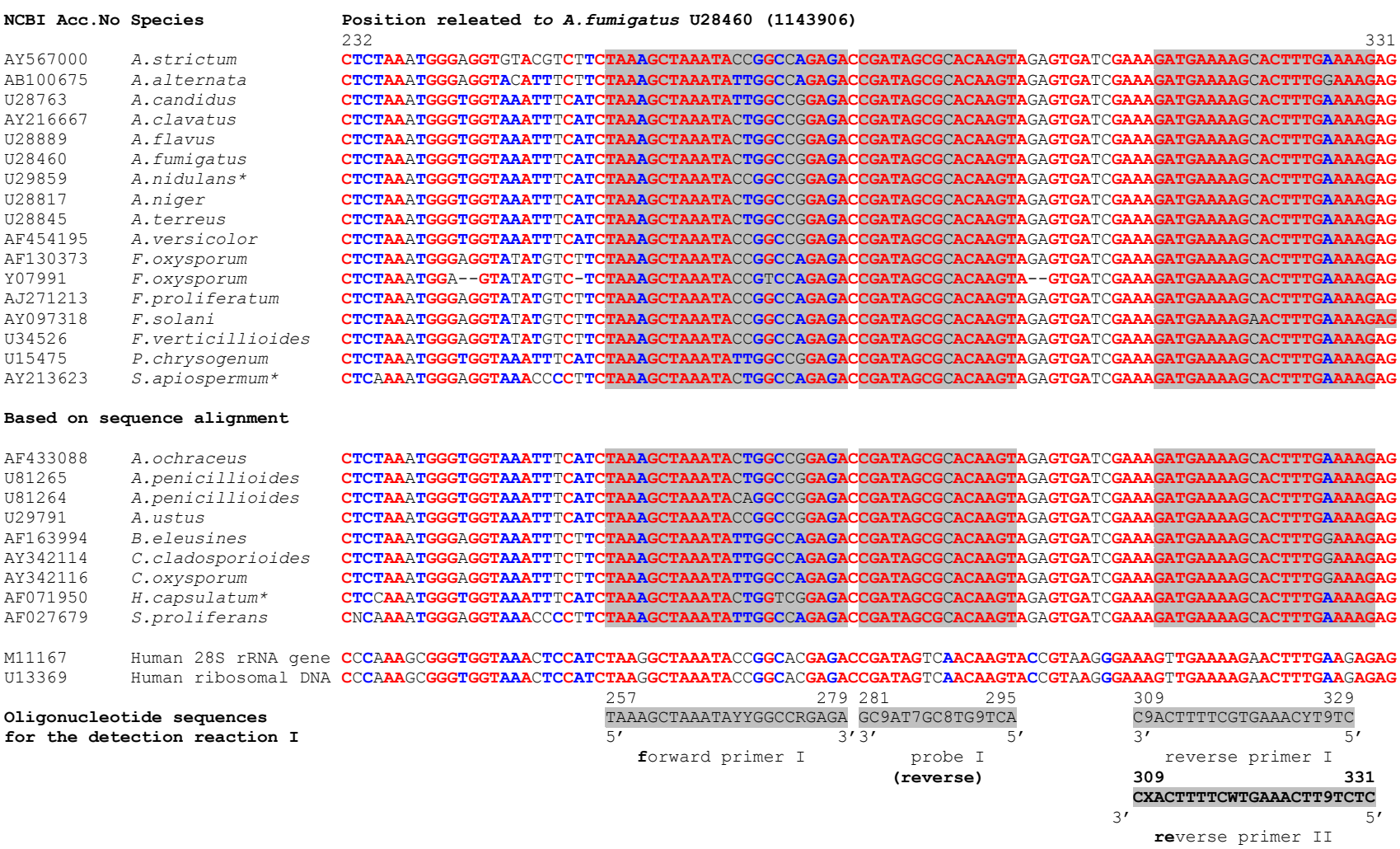


Figure 2.2.1

Panfungal detection reaction II

Based on experimental evidence

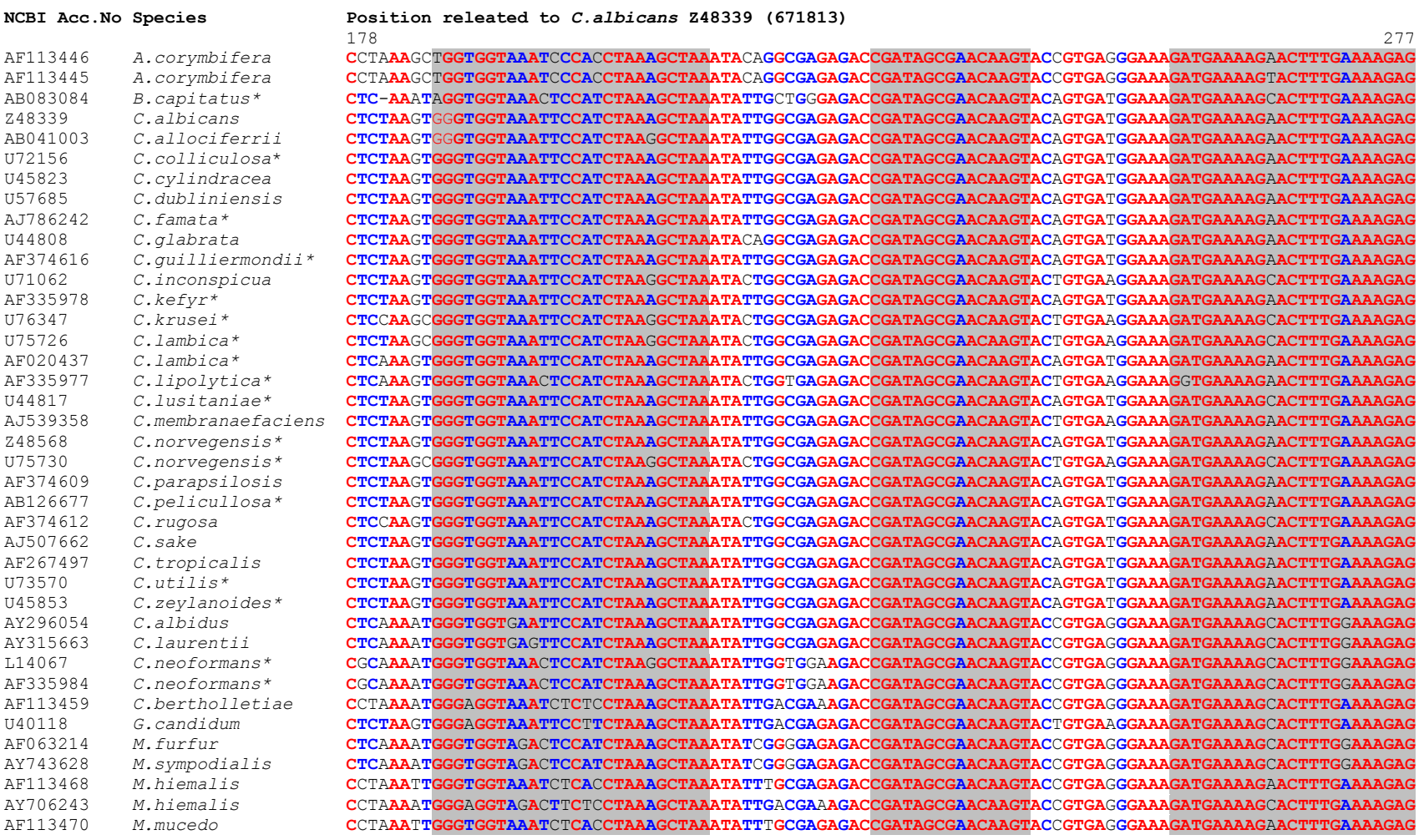
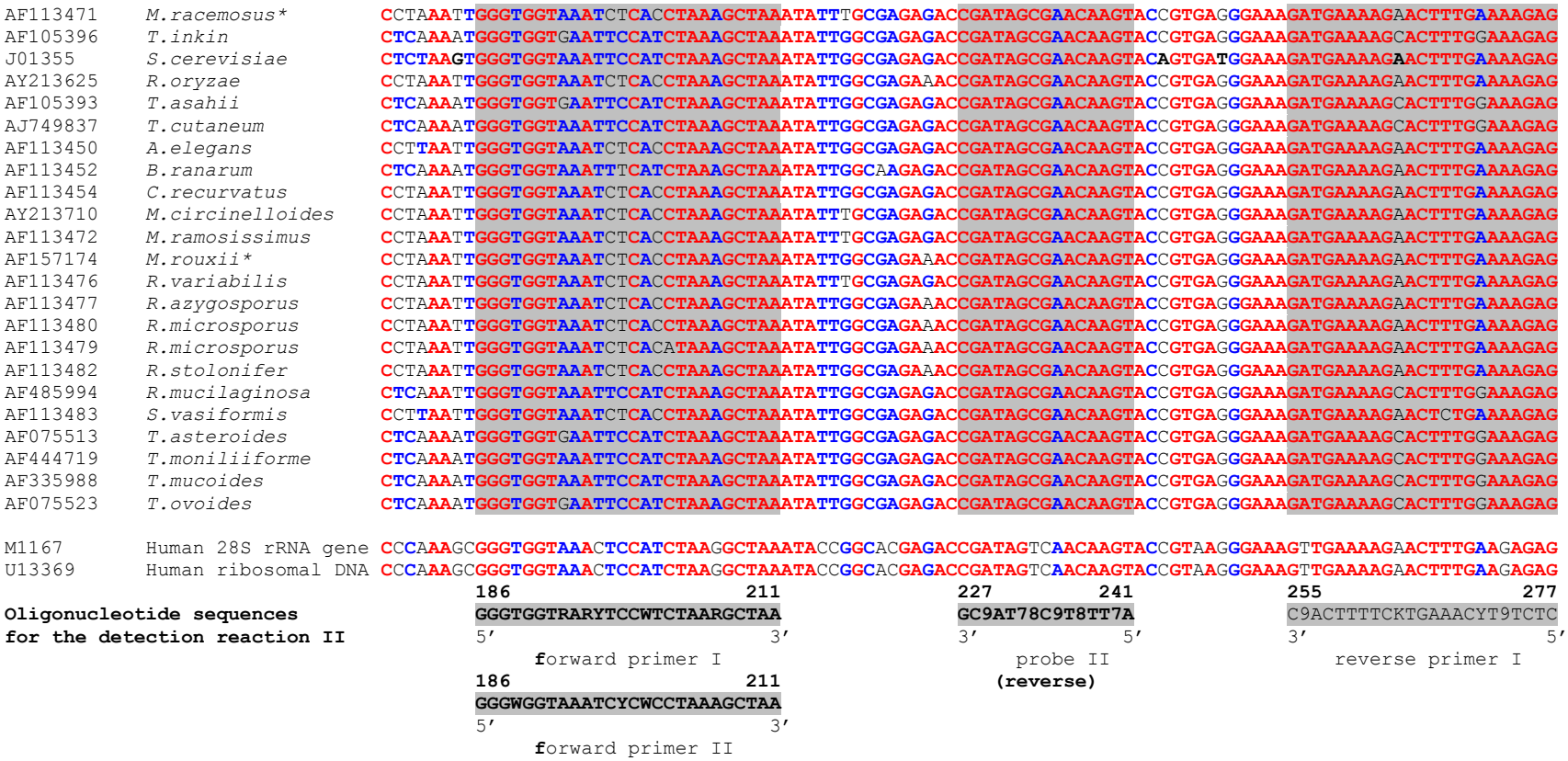


Figure 2.2.1. *(Continued)*

Based on sequence alignment



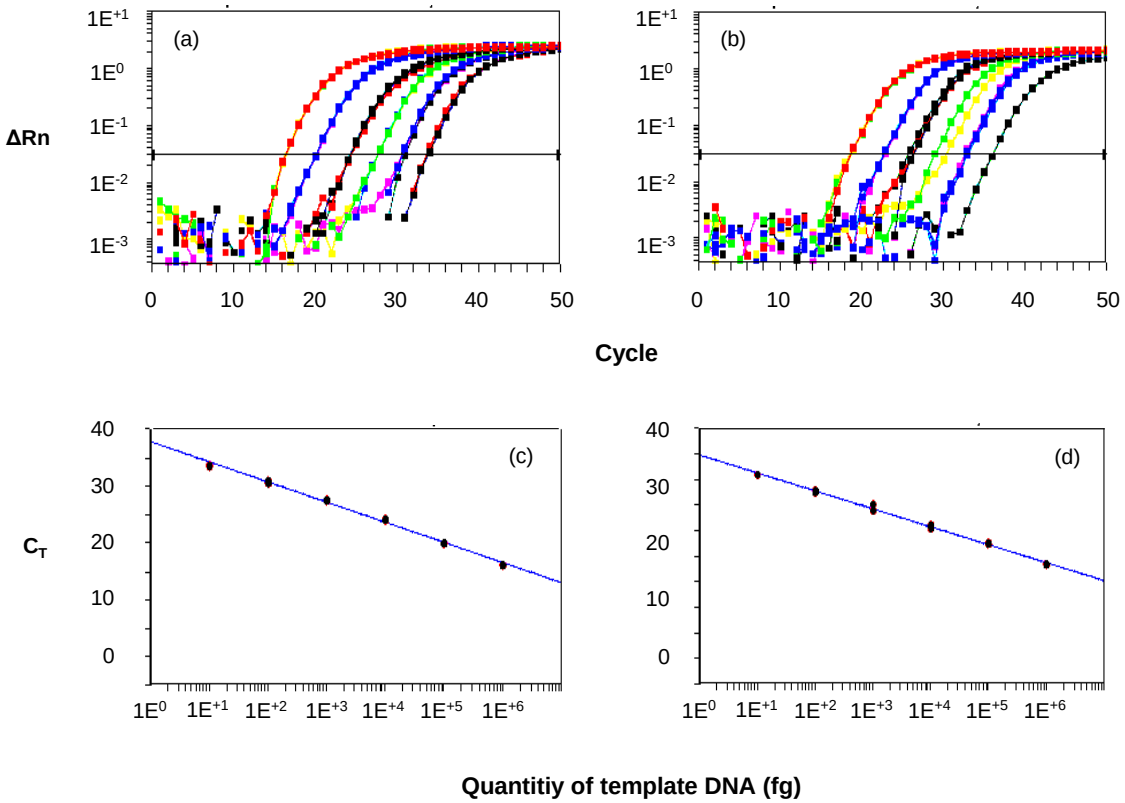
Legend:

Wobble positions used: R = G or A; W = A or T; Y = C or T; K = G or T
LNA modified nucleotides used: X = C-LNA or T-LNA; 7 = C-LNA; 8 = G-LNA; 9 = T-LNA

Figure 2.2.1. *(Continued)*

*** Species listed under other name in the NCBI database:** *Aspergillus nidulans* = NCBI *Emericella nidulans*, *Scedosporium apiospermum* = NCBI *Pseudallescheria boydii*, *Histoplasma capsulatum* = NCBI *Ajellomyces capsulatus*, *Candida colliculosa* = NCBI *Torulaspora delbrueckii*, *Candida famata* = NCBI *Debaryomyces hansenii*, *Candida guilliermondii* = NCBI *Pichia guilliermondii*, *Candida kefyr* = NCBI *Kluyveromyces marxianus*, *Candida krusei* = NCBI *Issatschenkia orientalis*, *Candida lambica* = NCBI *Pichia fermentas*, *Candida lipolytica* = NCBI *Yarrowia lipolytica*, *Candida lusitaniae* = NCBI *Clavispora lusitaniae*, *Candida norvegensis* = NCBI *Pichia norvegensis*, *Candida pelliculosa* = NCBI *Pichia anomala*, *Candida utilis* = NCBI *Pichia jadinii*, *Candida zeylanoides* = NCBI *Candida krissii*, *Blastoschizomyces capitatus* = *Dipodascus capitatus*, *Cryptococcus neoformans* = NCBI *Filobasidiella naoformans*, *Mucor racemosus* = NCBI *Rhizomucor racemosus*, *Mucor rouxii* = NCBI *Amylomyces rouxii*, *Geotrichum candidum* = NCBI *Galactomyces geotrichum*

Figure 2.2.1. *(Continued)* Multiple sequence alignment (Corpet F, 1988) of the highly conserved 28S rDNA gene region selected for a design of the panfungal detection system. About 90 different sequences accessible on the NCBI database were aligned including a variety of mould-, yeast- and the corresponding human sequences. Two RQ-PCR assays were designed to cover the indicated spectrum of fungal species. Detection reaction I covers moulds (21 isolates/strains tested experimentally and 8 species based on the sequence alignment) and detection reaction II covers primarily yeasts and Zygomycetes (42 isolates/strains tested experimentally and 16 species based on sequence alignment). The primer pairs and the probes (grey boxes) were placed within the target region. The indicated nucleotide positions refer to the sequences of *A. fumigatus* (NCBI accession no. U28460) for reaction I and *C.albicans* (NCBI accession no. Z48339) for reaction II, respectively. The asterisks indicate species that are listed under other names in the NCBI database.



Legend:

ΔR_n is the difference between emission intensity of reporter day in PCR with template and emission intensity of reporter day in PCR without template or in early cycles of a real-time reaction related to emission intensity of passive refference day
C_T threshold cycle is the number of cycles at which the fluorescence exceeds the threshold

Figure 2.2.2. Amplification plots (a, b) and standard curves (c, d) for quantitative fungus analysis by the panfungal detection system. The upper panels show amplification plots of serial logarithmic dilutions of fungal gDNA (ranging from 10 fg to 100 pg) derived from *A.fumigatus* (a) amplified by the panfungal detection reaction I and *C.albicans* (b) amplified by the panfungal detection reaction II. The lower panels (c, d) reveal the corresponding standard curves generated on the basis of the amplification profiles displayed. The assays underlying the generation of standard curves were performed in duplicates. The Y-intercept, which corresponds to the theoretical limit of detection, was determined at C_T 39,9 for reaction I and C_T 37,8 for reaction II. In view of the great similarity between the amplification profiles of all fungal species covered by the panfungal assay, the standard curves shown are applicable to the quantification of any *Candida*, *Aspergillus* and other fungal species detected.

2.3 Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region

2.3.1 AIM OF THE STUDY

The rapid identification of fungal pathogens in clinical specimens is a prerequisite for timely onset of the most appropriate treatment. The aim of the present study was to develop a sensitive method facilitating the identification of a broad spectrum of fungal species with a detection limit adequate for routine clinical diagnosis of IFDs in immunosuppressed individuals. The intended clinical application of the technique is the rapid identification of fungal species in clinical specimens that have been previously shown to be positive for fungal DNA by a broad-spectrum screening technique not capable of identifying the fungal species.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Fungal strains, bacteria, and viral isolates

The fungal reference strains tested were obtained from the ATCC (Rockville, USA), the DSM (Braunschweig, Germany), the IHMM, (Medical University of Vienna, Austria), and from the Faculty of Medicine, Charles University (FMCU), (Hradec Kralove, Czech Republic). All fungal strains were grown on Sabouraud dextrose agar, yeasts at 37°C (*Cryptococcus* spp. at 30°C) for 48 h and moulds at 30°C for one to seven days until sporulation. Yeast identification was performed by ID32 C test (BioMerieux, Marcy l'Etoile, France) and by micromorphology on rice-agar. Moulds were identified based on conventional macro- and microscopic morphological investigation. The reference strains are indicated in Table 2.3.1.
In addition, a panel of bacterial and viral microorganisms was selected for testing of cross-reactivity as indicated in Baskova et al. 2007.

2.3.2.2 Clinical materials

Fungus-positive clinical specimens including biopsies of pulmonary infiltrations, bronchotracheal secretions, peripheral blood, cerebrospinal fluid, and plasma were obtained from the St. Anna Children's Hospital, Vienna, Austria and from the Division of Clinical Microbiology, IHMM, Medical University of Vienna. Ocular specimens from patients with eye infections were provided by the FMCU, Hradec Kralove, Czech Republic. All clinical specimens were obtained from the patients after informed consent. Peripheral blood specimens from healthy volunteer donors were used as negative controls.

2.3.2.3 DNA extraction

The isolation and purification of DNA from fungal strains, as well as of fungal human, bacterial and viral DNA from different clinical materials was performed essentially as described earlier (Baskova et al. 2007).

a) Plasma containing white blood cells

Peripheral blood specimens anticoagulated with EDTA were kept at 4°C for at least 4 h to sediment the red blood cells. The entire supernatant, i. e. plasma containing white blood cells, was used for DNA extraction. The samples were centrifuged at 15.000 g for 10 min. Most of the supernatant was removed, leaving a residual volume of 100 µl, and 430 µl of LLB was added. The DNA extraction from other clinical materials was performed as described by Baskova et al. 2007.

b) Cerebrospinal fluid

The samples were centrifuged at 15.000 g for 10 min. Most of the supernatant was removed, leaving a residual volume of 100 µl, and 430 µl of LLB was added. The DNA extraction was performed as described above.

2.3.2.4 PCR amplification

a) First amplification round

The universal primers, ITS4 reverse primer (Turenne et al. 1999) (5'-TCC TCC GCT TAT TGA TAT GCT-3') and a newly designed forward primer (5'-TTT CAA CAA YGG ATC TCT TGG-3'), which we have termed ITS7 were used to amplify across the entire ITS1, 5.8S, ITS2, and part of the 18S and 28S regions of the rDNA gene. The 25 µl PCR reaction mix contained GeneAmp^{R} 1 x PCR buffer II (AB, Branchburg, New Jersey, USA), 2.5 mM $MgCl_2$ (AB), 200 µM deoxynucleotide triphosphates (dNTPs), dATP, dCTP, dGTP, and a 1:8 ratio of dUTP to dTTP (Invitrogen), 400 nM of each primer, 0.25 U heat-labile UNG (Hoffmann-La Roche Ltd, Basel, Switzerland), 2.5 U AmpliTaq® DNA Polymerase (AB), molecular biology grade water (Eppendorf, Hamburg, Germany) and 5 µl template DNA. The PCR was performed according to the following protocol: 10 min at 37°C (UNG activation), 10 min at 95°C (polymerase activation) and 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 50 s, and a final extension step at 60°C for 45 min, followed by cooling to 4°C (Longo et al. 1990).

b) Second amplification round

The primer used included the reverse primer ITS4 (sequence indicated above) and two fluorescein-labeled forward primers ITS86-I (5'-TGA ATC ATC GAR TCT TTG AAC G-3') and ITS86-II (5'-TGA ATC ATC GAG TTC TTG AAC G-3'), which hybridize to the 5.8S region of the rRNA gene. The PCR reaction mix contained 3 µl of the first round PCR product, GeneAmp^{R} 1x PCR buffer II (AB), 2.5 mM $MgCl_2$ (AB), 200 µM dNTPs (including dUTP), 400 nM of each primer and 1 U of AmpliTaq$^{®}$ DNA Polymerase (AB) in a total volume of 25 µl. The amplification conditions were identical to the first round of amplification, with omission of the initial UDG activation step. In laboratories less experienced with PCR work, the risk of contamination

inherent in nested PCR approaches can be reduced e.g. by adapting a single-tube two-round PCR technique described earlier (Trka et al. 1995).

2.3.2.5 Fragment analysis by capillary electrophoresis

Fragment analysis was performed on the ABI PRISM® 3100-Avant (AB) genetic analyzer, an automated fluorescence capillary electrophoresis system. The set-up of the instrument was done according to the manufacture's instructions. Due to the fluorescein label of the ITS86 primers, the matrix kit PowerPlex® Matrix Standards, 3100 (Promega, Mannheim, Germany) was used to establish a matrix file (dyset Z). Analysis parameters and length standards were established according to the PowerPlex® 16 System technical manual (Promega). 1 µl of the PCR product generated by semi-nested PCR amplification was mixed with 1 µl of the internal length standard ILS 600 (Promega) and 24 µl deionized formamide HiDi (AB). After denaturation for 3 min at 94°C and cooling to 4°C, the whole sample was injected into a 36 cm capillary column containing the high performance polymer POP-4™ (AB). Electrophoresis parameters on the ABI PRISM® 3100-Avant were set at 15 s injection time, 3 kV injection voltage, 15 kV electrophoresis voltage, and an oven temperature of 60°C. The average electrophoresis time was 30 min to permit detection of PCR fragments up to 500 nt in length. PCR product lengths were analyzed using the ABI PRISM® GeneScan® Analysis software 3.7 (AB). Signals were regarded as positive if the fluorescence intensity was higher than 50 reference fluorescence units (RFUs).

A number of precautions were undertaken to control the occurrence of false positive results. Multiple no-template and non-homologous template controls were processed together with the specimens tested.

2.3.3 RESULTS

2.3.3.1 Analysis of fungal reference strains

To determine the ability of the technique presented to identify a broad spectrum of human pathogenic fungi, a total of 96 reference strains derived from 60 different fungal species were analyzed by the semi-nested pan-fungal PCR amplification followed by capillary electrophoresis (Table 2.3.1). The most common strains are indicated in Table 2.3.1. Upon comprehensive sequence alignment of the targeted ITS2 region from all fungal isolates represented in the GenBank database, minor intra-species variations became apparent. However, when testing multiple isolates per fungal species, which were obtained from different sources, the ITS2 sizes observed corresponded well with those of the reference strains displayed in Table 2.3.1 (data not shown).

To verify the morphological classification of the fungal reference strains, the PCR products of each strain were sequenced and compared to the GenBank entries. In some instances, the sequence analysis data differed from the species designation provided by the supplier (the sequence of *Malassezia furfur* DSM 6171 corresponded with that of *Malassezia sympodialis* in the GenBank database, and the sequence of *C. sake* DSM 70763 with *C. oleophila*). The ITS2 amplicon length of all reference strains was determined by two independent PCR assays followed by four separate runs of capillary electrophoresis on the ABI PRISM 3100 genetic analyzer. From these measurements, the mean ITS2 fragment length was calculated for every fungal strain tested. Under standardized conditions, the run-to-run variation between amplicon size measurements revealed a 95% confidence interval of 0.7 nucleotide(s) (nt) in the case of *A. terreus* DSM 1958 (Table 2.3.1), thus demonstrating the high reproducibility of PCR amplicon size analysis. Unequivocal identification of individual fungal strains was possible in most instances, but certain limitations of the technology have become apparent, as discussed below. The sizes of ITS2 amplicons were in a range between 184 nt (*C. lipolytica*) and 381 nt (*Absidia corymbifera*) (Table 2.3.1).

The specificity of the assay for the detection of fungal target sequences was assessed by testing human genomic DNA extracted from peripheral blood of voluntary healthy donors and different viral and bacterial strains (see Materials and Methods). No cross-reactivity of the amplification and detection system with non-fungal targets has been observed (data not shown).

2.3.3.2 Sensitivity of the assay

The limit of detection was determined by testing serial dilutions of genomic fungal DNA derived from *A. fumigatus* and *C. albicans* as representatives of moulds and yeasts, respectively. Template DNA ranging from 1 fg to 1 pg was amplified by the semi-nested PCR protocol and products were evaluated on the ABI PRISM 3100 genetic analyzer. The reproducible detection limit of capillary electrophoresis after semi-nested PCR amplification was 1 fg for both fungal strains tested (Figure 2.3.1), which is equivalent to a fraction of a single fungal genome on the basis of the estimated genome mass of 32 fg for *Aspergillus* species, and 37 fg for *Candida* species.

2.3.3.3 Assessment of clinical applicability

To assess the ability of the assay to facilitate identification of specific fungal pathogens in the clinical setting, different types of specimens collected from 26 patients with documented fungal infection were investigated. The specimens tested included peripheral blood, plasma, cerebrospinal fluid, ocular specimens, secretions from the respiratory tract, and lung biopsies. Extraction of fungal DNA, pan-fungal semi-nested PCR amplification, and analysis of the ITS2 fragments by capillary electrophoresis were performed as described above. In order to ensure the absence of cross-reactivity with human DNA or the occurrence of external contamination, DNA from whole blood of healthy volunteer donors was extracted and analyzed in parallel with the clinical specimens investigated (Figure 2.3.2, panel 9). All clinical specimens studied were positive for fungal DNA by PCR analysis, and

the respective species were identified by comparing the ITS2 fragment sizes obtained by capillary electrophoresis to external reference standards (Table 2.3.1; Figure 2.3.2). The fungal species identified in the clinical specimens analyzed are listed in Table 2.3.2. The cerebrospinal fluid, peripheral blood and plasma samples tested revealed *C. albicans*, *C. glabrata*, and *A. flavus*/*A. niger*, respectively. The lung biopsies showed infections by *A. fumigatus* and *C. lipolytica*. The secretion samples from the respiratory tract tested positive for *A. fumigatus* in most instances and for *Rhizopus oryzae* on a single occasion. In one of the respiratory secretion specimens, a mixed infection with *A. fumigatus* and *C. albicans* was identified. All ocular specimens investigated revealed an infection by *Fusarium oxysporum*, caused by a point source outbreak in the hospital ward from which all these specimens were derived. Examples of ITS2 PCR fragments representing different fungal pathogens detected in the clinical samples investigated are displayed in Figure 2.3.2. Since certain strains of *A. flavus* and *A. niger* showed nearly identical sizes of ITS2 PCR amplicons upon capillary electrophoresis, unambiguous identification of the pathogen present was achieved by subsequent sequencing. In all instances, PCR positive results were confirmed by an independent technique, which included either DNA sequencing, microbiological culture or a DNA hybridization technique.

2.3.4 DISCUSSION

We have established a semi-nested PCR technique for amplification of the variable ITS2 region from the multi-copy ribosomal rRNA gene coupled with automated fragment length analysis to facilitate rapid species-specific identification of a large spectrum of clinically relevant fungal pathogens. The technique presented was specifically designed to permit identification of the fungal species in any clinical specimen previously shown to be positive for fungal DNA by a broad-spectrum amplification assay lacking the ability of species recognition (Klingspor and Jalal 2006; Basková et al. 2007; Costa et al. 2002; Maaroufi et al. 2004; White et al. 2003). Several PCR-based methods for species-specific identification exploit

the sequence variability of the ITS2 region, which is flanked by conserved sequences of the rRNA gene (Martin et al. 2000; Elie et al. 1998; Yamakami et al. 1996; Einsele et al. 1997; van Burik et al. 1998; Sandhu et al. 1995). The identification of cultured fungal strains by ITS2 fragment length analysis has been described previously (Chen et al. 2000; Turenne et al. 1999; Williams et al. 1995). However, the achievable detection limit of these approaches could render the detection of IFDs in routine clinical diagnosis difficult, because the concentration of fungal pathogens in specimens, such as peripheral blood, is generally very low (Loeffler et al. 2000). It is important to consider that in immunosuppressed patients the presence of fungal pathogens, even at extremely low concentrations, may reflect a life-threatening infection. We have established a pan-fungal semi-nested PCR protocol for efficient amplification of the fungal ITS2 regions using a newly designed and optimized forward primer for the first round of amplification. Our modification of the detection system based on the semi-nested amplification of the ITS2 region facilitates highly sensitive species identification, which renders the technique suitable for clinical application, especially in immunocompromised patients where conventional diagnostic methods often fail owing to low sensitivity or specificity. The lowest amount of fungal genomic DNA permitting consistent species recognition by the approach presented was 1 fg, which is equivalent to a fraction of a single fungal organism on the basis of the estimated genome mass (*Aspergillus* 32 fg, and *Candida* 37 fg) (Figure 2.3.1).

The PCR fragment lengths determined by capillary electrophoresis differed from those expected on the basis of sequence analysis and from previously published data (De Baere et al. 2002; Turenne et al. 1999). This divergence can be attributed primarily to the specific properties of the capillary electrophoresis apparatus used and to migration shifts during electrophoresis caused by the fluorescent dye component. Potential sources of intrinsic variability of fragment size measurements were compensated for by implementing an internal laboratory reference panel of well defined fungal strains. The method presented not only permits the identification of almost all clinically relevant species from the genera *Aspergillus* and *Candida*, which still account for the vast majority of IFDs, but also

facilitates the identification of a large panel of emerging fungal species, such as *Fusarium*, *Trichosporon*, or Zygomycetes. The ability to identify a broad spectrum of fungal pathogens is essential with regard to the changing epidemiology of invasive mycoses and differences in the susceptibility pattern of individual fungal species to conventional and new antifungal agents.

Of the clinical samples analyzed, ITS2 fragment length analysis permitted unequivocal identification of the fungal species in all specimens, with a single exception (Figure 2.3.2) attributable to the fact that some fungal species have similar or identical ITS2 fragment sizes (Table 2.3.1). From the perspective of optimal choice of treatment, the discrimination between pathogens is particularly important in instances in which the suspected fungal species require different antimycotic therapy (Slavin et al. 2004). Some pathogens, such as *A. flavus* (DSM818) and *A. niger* (ATCC10535, DSM737), which display similar ITS2 fragment sizes, share the susceptibility to identical antifungal agents, thus rendering unequivocal identification of the species less important (Mallié et al. 2005). Some other species that cannot be clearly discriminated by ITS2 fragment length analysis are, however, susceptible to different antifungal agents. This applies to *C. dubliniensis* (ATCC MYA-646) and *C. krusei* (IHMM, DSM 70075), the latter of which is intrinsically resistant to fluconazole (Messer et al. 2006), to *A.terreus* and *Trichosporon* spp., and to *Acremonium strictum* and *A. terreus*. In these instances, the analysis of reference fungal strains in the same run can be helpful in discriminating between the fungal species. Additionally, alternative methods, such as sequencing of the PCR product or restriction fragment length polymorphism (RFLP) analysis (Dendis et al 2003; Trost et al. 2004) may be instrumental in facilitating unequivocal identification of the fungal species present and permit the administration of adequate antifungal therapy.

A limitation of ITS2 fragment length analysis is the potential occurrence of intra-species variations of ITS2 fragment sizes in clinical isolates which do not correspond to the ITS2 sizes of the defined fungal strains presented in the reference panel. The testing of multiple isolates derived from the fungal species of interest revealed ITS2 sizes which correlated very well with those presented in Table 2.3.1.

Based on comprehensive ITS2 sequence alignments of clinical isolates represented in the GenBank database (up to 190 entries per species - in the case of *Saccharomyces cerevisiae*), the most common fragment sizes are indicated in the reference panel. However, it may be necessary to supplement or slightly modify the panel based on regional differences in the predominant occurrence or the emergence of certain fungal strains.

An essential feature of the technique presented is the ability to detect co-infections with different fungi by revealing two or more amplification signals of different size in a single electropherogram (Figure 2.3.2, panel 5). The identification of co-infections with different fungal pathogens, which is not uncommon in immunocompromised individuals, is an important prerequisite for the most appropriate antifungal therapy.

Our data demonstrate that fragment length analysis by capillary electrophoresis after semi-nested PCR amplification of the ITS2 region permits highly sensitive and reliable identification of a large panel of different potentially pathogenic fungi. The method is readily applicable in the routine laboratory practice and could contribute to successful management of IFDs.

Table 2.3.1. Mean fragment size (nt) with 95% confidence intervals of PCR amplification products of the ITS2 region from various fungal strains

Species	Strain	PCR fragment length (nt)	95% CI
Candida lipolytica	DSM 8218	183,4	183,3-183,5
Candida lipolytica	IHMM	184,0	183,9-184,1
Geotrichum candidum	DSM 1240	188,7	188,6-188,8
Candida lusitaniae	DSM 70102	196,3	196,2-196,4
Candida lusitaniae	IHMM	196,6	196,4-196,8
Candida pararugosa	IHMM	214,2	213,9-214,5
Candida rugosa	FMCU	214,3	214,2-214,4
Candida lambica	DSM 70090	241,3	240,7-241,9
Candida lambica	IHMM	243,1	242,7-243,5
Blastoschizomyces capitatus	IHMM	245,6	245,5-245,7
Candida parapsilosis	ATCC 22019	249,5	249,3-249,7
Candida parapsilosis	IHMM	249,6	249,4-249,8
Candida parapsilosis	DSM 11224	249,6	249,4-249,8
Candida norvegensis	FMCU	262,7	262,5-262,9
Candida norvegensis	DSMZ 70760	264,9	264,8-265,0
Candida tropicalis	DSM 5991	266,1	266,0-266,2
Candida valid	IHMM	266,3	266,3
Candida inconspicua	DSM 70631	266,6	266,5-266,7
Candida tropicalis	ATCC 750	266,8	266,7-266,9
Fusarium oxysporum	DSM 2018	273,9	273,8-274,0
Fusarium verticilloides	DSM 62264	275,5	275,3-275,7
Candida albicans	DSM 1386	277,5	277,5
Candida albicans	IHMM	277,7	277,6-277,8
Candida dubliniensis	IHMM	280,2	280,2
Candida krusei	ATCC 6258	281,6	281,4-281,8
Candida krusei	IHMM	282,1*	281,7-282,5
Candida dubliniensis	ATCC MYA-646	282,3*	282,2-282,4
Candida krusei	DSM 70075	282,3*	282,1-282,5
Aspergillus candidus	IHMM	283,6	283,5-283,7
Aspergillus candidus	DSM 814	283,8	283,5-284,1
Alternaria alternata	DSM 62006	284,0	283,7-284,3

Table 2.3.1. (*Continued*)

Species	Strain	PCR fragment length (nt)	95% CI
Fusarium proliferatum	DSM 840	284,9	284,8-285,0
Fusarium proliferatum	IHMM	284,9	284,8-285,0
Aspergillus fumigatus	ATCC 36607	285,5	285,0-286,0
Aspergillus fumigatus	DSM 819	285,5	285,2-285,8
Aspergillus fumigatus	IHMM	285,7	285,4-286,0
Aspergillus nidulans	DSM 946	285,9*	285,3-286,4
Aspergillus versicolor	IHMM	286,3	286,2-286,4
Aspergillus nidulans	DSM 820	286,3*	286,2-286,4
Aspergillus nidulans	IHMM	286,4*	286,2-286,6
Aspergillus clavatus	DSM 816	286,5*	285,9-287,1
Aspergillus clavatus	IHMM	286,8	286,7-286,9
Aspergillus versicolor	DSM 1943	287,7	287,4-288,0
Fusarium solani	DSM 62413	288,3	288,1-288,5
Aspergillus niger	ATCC 10535	288,4*	288,1-288,7
Aspergillus flavus	DSM 818	288,5*	287,9-289,1
Aspergillus niger	DSM 737	288,9*	288,7-289,1
Aspergillus glaucus	IHMM	289,0*	289,0
Aspergillus flavus	IHMM	289,6	289,5-289,7
Aspergillus niger	IHMM	291,2*	290,9-291,5
Acremonium strictum	DSM 3567	294,5*	294,4-294,6
Aspergillus terreus	DSM 1958	294,7*	294,0-295,3
Aspergillus terreus	IHMM	295,0	294,9-295,1
Cryptococcus humicolus	FMCU	295,3	295,2-295,4
Trichosporon inkin	IHMM	295,6*	295,3-295,9
Candida cylindracea	DSM 2031	295,8*	295,4-296,2
Aspergillus terreus	DSM 826	295,8*	295,7-295,9
Trichosporon beigelii	IHMM	295,9*	295,8-296,0
Trichosporon cutaneum	DSM 70707	296,0*	295,9-296,1
Trichosporon asahii	IHMM	297,3	297,1-297,5
Mucor hiemalis	DSM 2656	301,2	301,0-301,4
Candida utilis	DSM 2361	303,2	303,1-303,3
Cryptococcus laurentii	IHMM	303,5	303,2-303,8
Cryptococcus laurentii	DSM 70766	304,8	304,7-304,9

Table 2.3.1. (*Continued*)

Species	Strain	PCR fragment length (nt)	95% CI
Cunninghamella bertholletiae	IHMM	306,0	305,9-306,1
Candida allociferii	IHMM	306,5	306,4-306,6
Mucor racemosus	DSM 62760	308,4	308,2-308,6
Mucor rouxii	DSM 1191	310,1	309,9-310,3
Candida pelliculosa	FMCU	311,1	310,9-311,3
Cryptococcus neoformans	DSM 70219	313,7	313,6-313,8
Cryptococcus neoformans	IHMM	313,7	313,6-313,8
Candida zeylanoides	DSM 70185	313,9	313,8-314,0
Candida zeylanoides	IHMM	313,9	313,8-314,0
Candida pelliculosa	DSM 70130	314,5*	314,4-314,6
Candida sake	DSM 70763[a]	314,5*	314,4-314,6
Candida membranifaciens	DSM 70109	315,7	315,6-315,8
Rhizopus oryzae	DSM 853	316,2	316,2
Rhizopus oryzae	IHMM	316,2	316,1-316,3
Candida guilliermondii	DSM 70051	318,7	318,4-319,0
Candida guilliermondii	DSM 11947	318,8	318,6-319,0
Candida guilliermondii	IHMM	318,8	318,6-319,0
Candida famata	IHMM	320,8	320,7-320,9
Candida famata	DSM 70590	321,0	320,9-321,1
Scedosporium apiospermum	ATCC 28206	321,7	321,4-322,0
Mucor mucedo	DSM 809	346,6	346,4-346,8
Cryptococcus albidus	DSM 70215	348,2	348,1-348,3
Candida glabrata	ATCC 2001	356,3	356,2-356,4
Candida glabrata	IHMM	358,3	358,2-358,4
Candida glabrata	DSM 1122	358,6	358,5-358,7
Malassezia furfur	DSM 6171[b]	359,2	359,2
Saccharomyces cerevisiae	IHMM	362,0	361,9-362,1
Saccharomyces cerevisiae	DSM 70449	362,9	362,6-363,2
Candida kefyr	DSM 70073	371,0	370,8-371,2
Candida kefyr	IHMM	371,0	370,9-371,1
Absidia corymbifera	IHMM	380,8	380,5-381,1
Candida colliculosa	IHMM	386,4	386,2-386,6

Table 2.3.1. (*Continued*)

[a] Determined as *Candida oleophila* by sequence analysis
[b] Determined as *Malassezia sympodialis* by sequence analysis

It should be noted that the similarity of fragment sizes between individual fungal species, as indicated in the Table (*), may require further investigation (e.g. sequencing) to clearly identify the species present. In the setting of IFI, this may be relevant for species susceptible to different antifungal agents.

Table 2.3.2. Clinical specimens analyzed by ITS2 fragment length analysis

Specimen *(n)*	**ITS2 fragment sizes**	**Species identification by ITS2 fragment length analysis**	**Confirmation**
Bronchotracheal secretions (9)	285.2- 285.6	*A. fumigatus*	culture
Bronchotracheal secretion (1)	316.2	*Rhizopus oryzae*	culture
Bronchotracheal secretion (1)	277.5	*C. albicans*	DNA hybridization culture
	285.3	*A. fumigatus*	
Lung biopsy (1)	285.3	*A. fumigatus*	DNA sequencing
Lung biopsy (1)	184.1	*C. lipolytica*	DNA sequencing
Ocular specimens (10)	273.9 - 274.0	*Fusarium oxysporum*	DNA sequencing, culture
Peripheral blood (1)	358.1	*C. glabrata*	culture
Plasma (1)	288.5	*A. flavus / A. niger*	DNA sequencing (*A. flavus*)
Cerebrospinal fluid (1)	277.4	*C. albicans*	culture

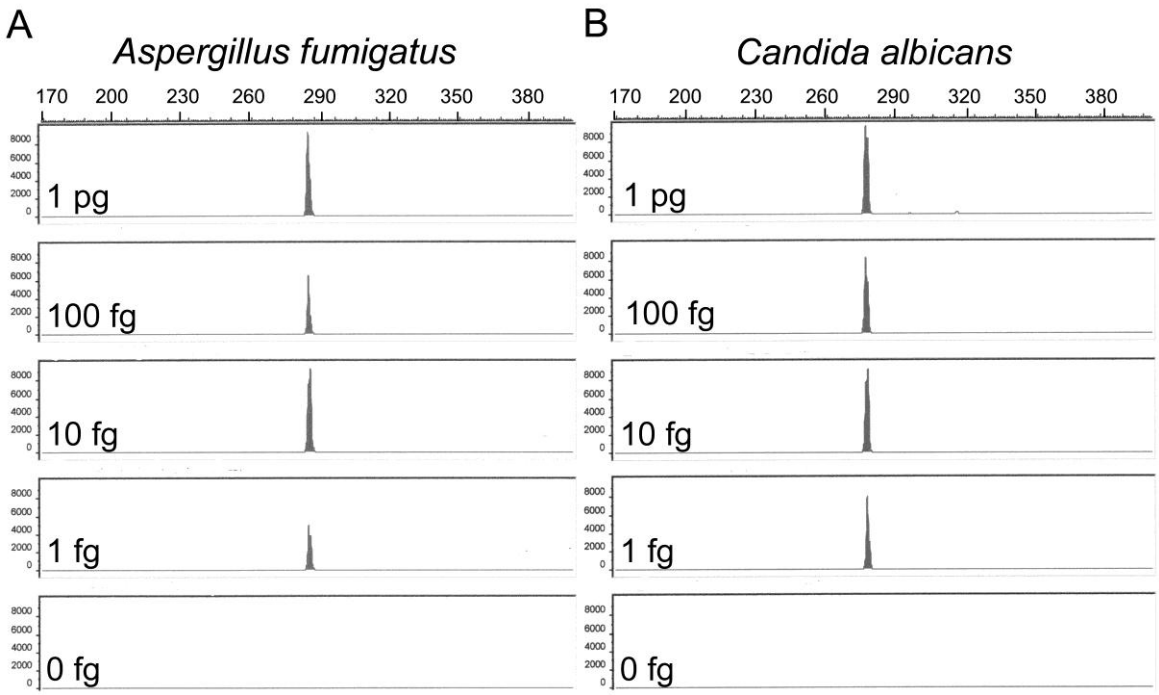
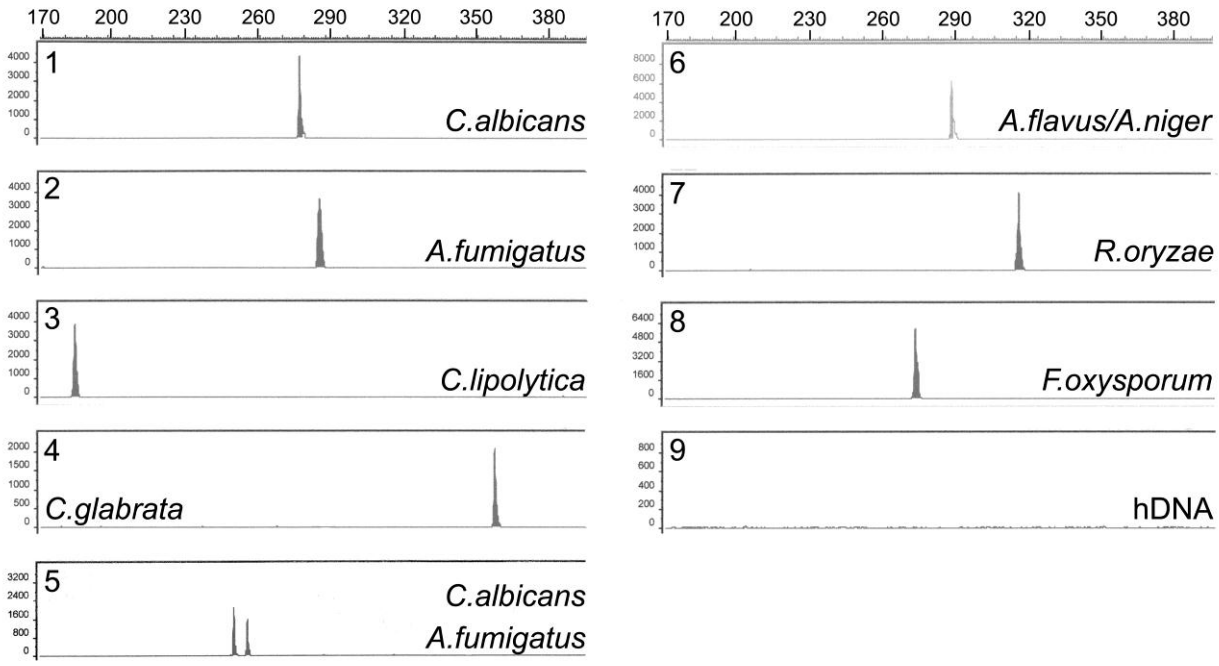


Figure 1. Sensitivity assessment of fragment analysis. Serial dilutions of genomic DNA from *A. fumigatus* and *C. albicans* (1 pg, 100 fg, 10 fg, 1 fg and 0 fg) were used as templates for semi-nested PCR amplificationof the ITS2 region. The PCR products were evaluated by capillary electrophoresis on the ABI PRISM® 3100-Avant Genetic Analyzer



No.	Specimens	Peak Size	Reference Size	Reference Species
1	cerebrospinal fluid	277.4	277.5 ± 0.1	*Candida albicans*
2	lung	285.3	285.5 ± 0.3	*Aspergillus fumigauts*
3	lung	184.1	184.0 ± 0.1	*Candida lipolytica*
4	blood	358.1	358.3 ± 0.2	*Candida glabrata*
5	plasma	288.5	288.5 ± 0.6 or 288.4 ± 0.3	*Aspergillus flavus/A.niger*
6	bronchotracheal secretion	277.5 and 285.3	277.5 ± 0.1 and 285.5 ± 0.3	*Candida albicans, A.fumigatus*
7	bronchotracheal secretion	316.2	316.2 ± 0.1	*Rhizopus oryzae*
8	ocular specimen	274.0	273.9 ± 0.1	*Fusarium oxysporum*
9	human DNA	--	--	--

Figure 2.3.2. Examples of internally transcribed spacer 2 (ITS2) fragment length analysis of fungal pathogens detected in clinical specimens. The analysis was performed on the ABI PRISM ® 3100-Avant Genetic Analyzer. Electrophoreograms of eight clinical cases are presented. The fungal species were identified by comparison of the detected PCR fragment sizes with the external reference panel. Sample 9 is a negative control of amplification (human DNA from a healthy volunteer donor)

3. CONCLUSION

As a consequence of the growing number of immunocompromised patients and the expanding spectrum of newly emerging fungal species, opportunistic IFDs will continue to be a major cause of morbidity and mortality in this population. Early detection of IFD in high-risk patients is a prerequisite for rational and timely initiation of effective antifungal therapy. Conventional techniques display limited sensitivity and/or specifity in detecting systemic fungal infections, and the results are mostly available late in the course of infection. Recent advances in the development of non-invasive and culture-independent diagnostic techniques have led to significant improvement by facilitating earlier detection of IFD by incorporating biomarkers and molecular technique results into diagnostic algorithms (Ostrosky-Zeichner 2012).

Although antigen detection has shown variable performance in large clinical studies revealing both benefits and limitations, the availability of standardized protocols and quality assurance provided by commercial kits have allowed their inclusion in the diagnostic criteria for IFD established by the EORTC/MSG Consensus Group. The potential value of nucleic acid-based approaches to the detection and identification of fungal pathogens in immunocompromised patients is indisputable, as shown by a number of studies. To date, a large number of different molecular techniques have been published including different PCR approaches. However, the current lack of standardization and validation across a wide spectrum of diagnostic laboratories has prevented inclusion of these tests in the diagnostic criteria of IFD proposed by the EORTC/MSG Consensus Group. For clinical implementation of reliable and robust molecular methods, the laboratory procedures must be internationally standardized and evaluated in large-scale clinical trials to permit definition of widely accepted PCR performance parameters and have to be available for routine use. In order to establish a firm basis for clinical validation of molecular testing, there is urgent need for large prospective studies combining these techniques with a variety of established diagnostic tools to determine the potential role of PCR-based methods in the clinical management of IFD.

The acceptance of molecular diagnostic strategies in future consensus criteria for the diagnosis of IFD would significantly impact on clinical mycology. The inclusion of molecular testing in routine diagnostic screening would provide a basis for timely detection and identification of the causative fungal pathogen, quantification of fungal load and monitoring of the response to treatment, thereby leading to better management and control of IFD. In this respect, the techniques described in the present thesis and their implementation in an international study of pediatric cancer patients can be regarded as an important step towards the achievement of the indicated goals.

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